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Can Arsenobetaine Act as a Methyl-Donor in the One Carbon Cycle?

MASTER'S THESIS

to achieve the university degree of

Master of Science

Master's degree programme: Chemistry

submitted to

Graz University of Technology

Supervisor

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Institute of Chemistry, University of Graz

Graz, April 2015

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Die vorliegende Arbeit wurde am Institut für Chemie - Bereich Analytische Chemie der Karl-Franzens Universität Graz in der Zeit von Oktober 2013 bis April 2015 unter der Leitung von Ao. Univ. Prof. Dr. Mag. Walter Goessler durchgeführt.

Abstract

Arsenobetaine (AB) is known to be the major arsenic compound in marine animals. Concentrations exceeding hundreds of mg As/kg fish have been observed. It is commonly accepted that AB shows no toxic effects even at these high concentrations. In various publications it is reported that AB is excreted with a half-life of about 7 hours via the kidneys without being metabolized.

Glycinebetaine, the nitrogen analog of arsenobetaine, is a well-known methyl donor in the one carbon cycle. We investigated whether AB is able to exchange a methyl-group as well.

Deuterated AB (d^9AB) was orally ingested by 5 volunteers and their urine was investigated whether deuterated methyl-groups from the d9AB were replaced by non-deuterated ones, using molecule-selective mass spectrometry (HPLC-ESMS). To enable an appropriate determination of AB, we tried to remove the urine matrix using solid phase extraction (SPE) and furthermore we developed an HPLC-ESMS method which is able to determine low concentrations of AB in urine samples directly. With our method no changes in the ingested d9AB could be observed and we conclude that less than 0.05 % of the excreted d9AB were metabolised.

We also determined the arsenic speciation in all urine samples with HPLC-ICPMS. We used cation-exchange and anion-exchange chromatography to ensure that no unknown arsenical was excreted. Both chromatoraphical methods led to the same results: In all urine samples the major arsenical was AB and in some samples low signals for inorganic As, DMA and MA, but no unknown arsenicals, could be observed.

Matrix effects easily influence the results of ESMS, so we investigated the effects of the urine matrix. We determined different urine compounds and their influence on the signal of AB. The biggest signal suppression of the tested compounds was caused by urea. Unfortunately we could not identify all compounds of the complex urine matrix and their influence in ESMS.

Besides As, the concentrations of 28 elements in the urine samples were determined with ICPMS. Concerning the total amount of excreted elements, differences between the 5 volunteers could be observed, depending on their eating and drinking behaviour. Linear correlations between excreted elements were found as well.

Zusammenfassung

Arsenobetain (AB) ist die Hauptarsenverbindung in Meerestieren und wurde in Konzentrationen bis zu hunderten mg As/kg Fisch gefunden. Auch in diesen hohen Konzentrationen wird es als nicht toxisch eingestuft und eine schnelle Ausscheidung über die Nieren, mit einer Halbwertszeit von ~7 Stunden, wurde berichtet.

Glycinbetain (GB), das Stickstoffanalogon zu AB, agiert im menschlichen Körper als Methylgruppendonor im Kohlenstoffcyclus. Wir untersuchten ob AB dieselben Transmethylierungen wie GB durchführen kann.

Für die Untersuchung nahmen 5 Freiwillige 2 mg As, in Form von deuteriertem AB (d^9AB), zu sich. Ihre Urinproben wurden mit molekülselektiver Massenspektrometrie (HPLC-ESMS) auf den eventuellen Austausch der deuterierten Methylgruppen des d9AB mit nicht deuterierten untersucht. Um AB besser in den Urinproben nachweisen zu können, wurde eine Aufreinigungsmethode mittels Fest-Phasen-Extraktion (SPE) verwendet. Später entwickelten wir eine Methode, mit der wir geringe Mengen an AB im Urin direkt mit HPLC-ESMS messen können. Mit dieser Methode konnte kein Austausch der Methylgruppen des d9ABs festgestellt werden und wir können festhalten, dass < 0,05 % des AB im menschlichen Körper metabolisiert wurden.

Weiters haben wir die Arsen-Speziation in allen Urinproben mittels HPLC-ICPMS bestimmt. Wir verwendeten Kationen- und Anionenaustauschchromatographie, um sicher zu gehen, dass keine unbekannten Spezies übersehen wird. Die beiden Methoden lieferten dieselben Resultate: In allen Urinproben war AB die Hauptarsenspezies und in manchen Proben konnten geringe Mengen an anorganischem Arsen, DMA und MA, jedoch keine unbekannten Arsenspezies, detektiert werden.

Da ESMS einem starkem Matrixeinfluss unterliegt, haben wir einzelne Bestandteile der Urinmatrix auf ihren Einfluss auf die Signalstärke des AB untersucht. Die stärkste Signalunterdrückung konnte mit Harnstoff festgestellt werden. Leider wurden nur wenige Bestandteile der Urinmatrix identifizieren und deren Einfluss auf die Signalstärke aufgeklärt.

Die Urinproben wurden auf die Konzentrationen von weiteren 28 Elementen mittels ICPMS untersucht. Hierbei konnten größere Unterschiede zwischen den einzelnen Personen beobachtet werden, da die ausgeschiedenen Mengen stark vom Ess- und Trinkverhalten beeinflusst werden. Zwischen den Konzentrationen einiger Elemente konnten starke lineare Korrelationen beobachtet werden.

Danksagung

Lieber **Walter**, bei dir möchte ich mich als erstes bedanken. Danke für die tolle Betreuung und das tolle Projekt. Danke für deinen Hang alles noch etwas besser zu machen; so sind sehr interessante Daten und eine ganz gute Arbeit entstanden. Weiters möchte ich mich bei dir noch sehr herzlich für die Möglichkeit bedanken an so vielen Tagungen nicht nur teilzunehmen sondern auch präsentieren zu dürfen.

Sarah, Jaqui, Oliver und Walter; an euch geht ein besonders großer Dank. Danke, dass ihr an meiner Studie teilgenommen habt, meinen Arsencocktail getrunken habt und 3,5 Tage lang mit einem Plastikkanister aufs Klo gegangen seid. Ohne euch hätte es meine Studie nicht gegeben!

Lieber **Markus**, keiner hat die Höhen und Tiefen der letzten 1,5 Jahre so stark gemerkt wie du. Ich danke dir, dass du mich nicht nur ausgehalten sondern immer bestmöglich unterstützt hast. Die Diskussionen mit dir waren oft sehr hilfreich und mittlerweile bist du schon ein ganz guter Analytiker geworden! Weiters möchte ich mich bei dir für das Berechnen der Fits bedanken.

Ein großer Dank geht an meine **Familie** und ganz besonders meine **Mama**. Ohne euch wäre ich nicht so weit gekommen und ich möchte mich bei euch für diese grenzenlose Unterstützung bedanken! Es ist super so viele Leute hinter sich zu wissen!

Ein herzlicher Dank geht an **Simone**, **Oliver**, **Kathi** und die ganze **ACHE-Arbeitsgruppe!** Danke für eure anregenden Diskussionen und eure Unterstützung, wenn einmal etwas nicht so gelaufen ist wie es sollte. Es arbeitet sich nirgendwo besser als in einem so starken Team!

Vielen Dank an **Jaqui**, **Hanna** und **Michi** für die Unterstützung bei den Probenvorbereitungen.

Abschließend möchte ich mich noch beim ganzen **Bereich der Analytischen Chemie** für das angenehme Arbeitsklima bedanken.

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1 Scope of this Master Thesis

- Development of a clean-up method with SPE to enable the measurement of low amounts of AB in urine with LC-ESMS
- Development of a HPLC-method for the measurement of low amounts of AB in urine with LC-ESMS without clean-up
- Improving the knowledge of the human urine matrix and its signal suppression in ESMS
- Investigate if AB can act as a methyl-donor in the one carbon cycle similar to GB
- Determination of the concentration of 29 elements in human urine and their correlations

2 Introduction

2.1 Arsenic

2.1.1 Basic facts

Arsenic occurs naturally mainly in the earth's crust. At room temperature elemental arsenic is a solid grey substance and it is chemically classified as a metalloid. In the environment it occurs usually trivalent or pentavalent bound. Most arsenic compounds are colourless and have no smell. Therefore, it is not possible to notice their presence [1]. Arsenic containing molecules can be classified in two categories. Compounds containing only arsenic, oxygen, chlorine, sulphur and hydrogen are called inorganic arsenicals. Arsenic molecules containing arsenic, carbon, hydrogen and other elements are called organic arsenicals. The different compounds containing arsenic are called arsenic species. There are big differences between them, for example their toxicity, which will be discussed in chapter *2.1.3 Species and toxicity* in more detail.

2.1.2 History of arsenic

Arsenic has a long famous history as poison and drug. Till now everybody knows a lot of the arsenic murder and many people misleadingly think that arsenic always has to be dramatically toxic. Until the middle of the 1850s arsenic was used as a poison to eliminate rivals. The nice thing about using arsenic was its availability, its cheapness and the fact that it is a white, odourless, non-tasting powder. Furthermore the most visible symptoms of acute arsenic poisoning, like vomiting, diarrhoea and horrible pain, could easily be thought to be from other common diseases from this time [2]. Advantageously there was no real detection method for arsenic till 1836. For this reason no real evidence of an arsenic poisoning could be demonstrated and it was used a lot for example by the Medici [3]. One of the most famous victims was Britanicus poisoned by Nero and the French even called it "powder of success" [4].

1836 James Marsh developed the first reliable analytical method for the detection of arsenic, which is still called "Marsh test". In this method arsenic gets converted to volatile arsine. Then it is heated and breaks down generating elemental arsenic which accumulates, as a silvery substance, on a cold glass plate. Since this time the number of arsenic poisoning decreased strongly [5].

However there is a positive site of arsenic too; for example its long history as drug. Already Hippocrates and Aristoteles used arsenic compounds against ulcers [6]. In the middle age many different arsenicals were used for the treatment of various diseases. There was another breakthrough at the beginning of the eighteen century with “Fowler’s Solution” which contains potassium arsenite. It was used against syphilis, asthma and rheumatism and was the first chemotherapeutic drug against leukaemia [7]. In the eighteen century about sixty different arsenic containing drugs were used as a cure-all [6]. 1910 Paul Ehrlich discovered the effect of arsphenamine against syphilis and it was used as Salvarsan for about 40 years until the discovery of penicillin [8]. Till now there are some arsenic containing drugs in use, for example arsenic trioxide against acute promyelocytic leukaemia [9] and they try to use it against different types of cancer too [10]. In some countries Melarsoprol, an arsenic containing drug as well, is still used against African trypanosomiasis (sleeping sickness) [4].

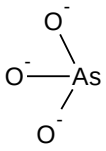
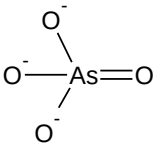
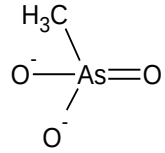
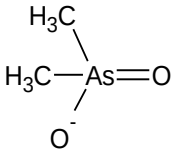
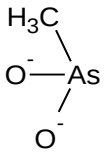
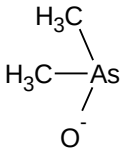
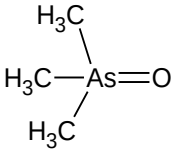
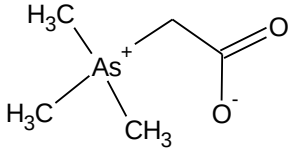
Besides its use as poison and drug arsenic played an important role in many different fields. In the 1800s copper acetoarsenite, also called “Paris Green” was used as a pigment in toys, candles and wallpapers. It did not take long, till the negative effects of “Paris Green” to the human health could be observed [11]. Afterwards it was used as insecticide till 1867. Other inorganic arsenicals, like lead arsenate or chromated copper arsenate, continued its use as a pesticide. Nowadays they are replaced by less toxic organic ones like sodium methanearsonate or dimethylarsinic acid (DMA). Another important use of arsenic was its cosmetic use [3]. Many people in the 19th century used for example Fowler’s solution as a cosmetic wash or other arsenicals as face powder, or rubbed them in their hair. In these days, the use of arsenic was correlated to beauty and freshness [4].

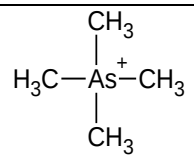
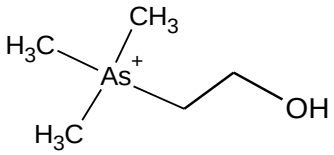
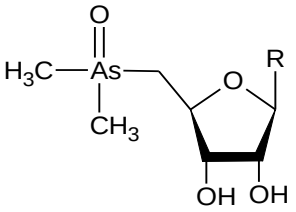
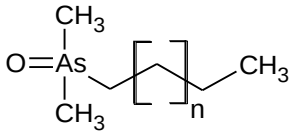
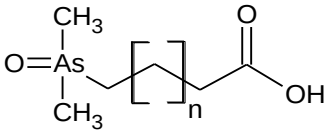
Concerning beauty and freshness obtained from arsenic, many people started eating it, to look healthy and beautiful without make up. The Styrian Arsenic Easters are probably the most famous example for this. Since the 17th century these people consumed about 130 mg of arsenic trioxide, two or three times a week [4]. Some of them ingested up to 400 mg per dose to enhance their beauty and health. The consumption of arsenic should help breathing easy, even during hiking in the mountains, help digesting heavy meals and increase courage and sexual potency [12]. Because of these benefits the people feed arsenic to their horses as well. Many of these people consumed high doses, more than double of the normal lethal dose, of arsenic over many years without dying. Till now nobody understands how they survived this. One approach is the bad bioavailability of arsenic trioxide powder but even by keeping this in mind the doses are really high and should have been lethal. Therefore, these people must have developed a significant tolerance to arsenic [12].

2.1.3 Species and toxicity

There are many different molecules containing arsenic. These molecules are called arsenic species and can be grouped in organic (containing As, C, H and many other elements) and inorganic ones (containing only As, H, O, S or Cl). Another way of classification is its binding form. Referring to this the arsenic species can be split in trivalent and pentavalent ones. The most common arsenic species are summarized, in their most deprotonated form, in Table 1.

Table 1 Common arsenic species [13, 14]

Name	Abbreviation	Structure	pK values
Arsenite	As(III)		pK_1 = 9.2 pK_2 = 12.1 pK_3 = 13.4
Arsenate	As(V)		pK_1 = 2.2 pK_2 = 7.0 pK_3 = 11.5
Methylarsonate	MA		pK_1 = 4.1 pK_2 = 8.7
Dimethylarsinate	DMA		pK = 6.2
Methylarsonite	MA(III)		
Dimethylarsinite	DMA(III)		
Trimethylarsine oxide	TMAO		
Arsenobetaine	AB		pK = 2.1

Tetrametylarsonium ion	TETRA	
Arsenocholine	AC	
Arsenosugars		
Arsenic containing hydrocarbons		
Arsenic containing fatty acids		

Because of its different toxicities it is very important to determine the arsenic species. Usually inorganic arsenicals are more toxic than the organic ones, and trivalent species are more toxic than the pentavalent ones. An overview of the different toxicities is given in Table 2.

Table 2 LD_{50} values of some arsenic species [1, 15, 16]

Arsenic species	**LD_{50} (oral) [mg / kg body mass]**
Arsenite	14-39[1], 15-145[2];
Arsenate	~20[1], 110-175[2]
MA	~100[3]; 700-1800[1]; 960-3200[2]
DMA	640-1400[2]; 700-2600[1]
AB, AC, TMAO	>10000[1]
TETRA	600-900[1]

[1] mouse; [2] rat; [3] rabbit

For adults the lethal dose of arsenic trioxide is about 100-200 mg [16], although the arsenic eaters survived higher doses.

In urine of unexposed humans typically less than 10 µg/L total arsenic can be found [17, 18]. The main species are DMA (60-80 %), MA (10-20 %) and inorganic arsenic (10-20 %) [19]. Therefore, the urine used for the clean-up experiments was spiked with As(III), As(V), MA, DMA and AB to simulate an urine after fish consumption. The intake of arsenic is mainly via

food, drinking water and sometimes via occupational exposure. Almost the total arsenic is excreted via the urine; hence urine analysis is the method of choice to study arsenic in humans [17].

Arsenobetaine

Arsenobetaine is the arsenic analogue to trimethylglycine, also called glycinebetaine (GB) or only betaine, which is an important osmolyte and methyl-donor in the human body.

It has been reported for many years that lots of marine animals contain really high amounts of probably nontoxic arsenic. About 50 years of research were needed to prove that the dominant arsenic species is arsenobetaine [20]. Until now, the AB content of many marine animals has been determined. Some concentrations can be found in Table 3.

Table 3 Total arsenic concentration and AB content in muscle tissues of marine animals [21, 22]

Animal	As [mg/kg wet weight]	AB [% of total As]
Elasmobranchs (fish)	3.1-44	≥94
Teleosts (fish)	0.1-170	48 to >95
Lobsters (crustaceans)	4.7-5.2	77 to >95
Pawns (crustaceans)	5.5-21	55 to >95
Crabs (crustaceans)	3.5-8.6	79 to >95
Bivalves (molluscs)	0.7-2.8	44 to 88
Gastropods (molluscs)	3.1-117	58 to >95
Cephalopods (molluscs)	~49	72 to >95

Beside marine animals some freshwater fish and fungi contain AB as their major arsenic species as well. Some examples are given in Table 4.

Table 4 Total arsenic concentration of freshwater fish and fungi, whereat AB is the major arsenic species [23, 24]

Animal		As [mg/kg]
Rainbow trout	Salmo gairdneri	1.5[1,3]
Japanese smelt	Hypomesus nipponesis	1.1[1,3]
Basidiomycetes	Agaricus subrutilescens	10.8 ± 0.4[2] (96 % AB)
Basidiomycetes	Albatrellus cristatus	7.7 ± 1.0[2] (91 % AB)

[1] mg/kg wet weight; [2] mg/kg dry mass; [3] values refer only to muscle tissue

The reason for the high concentrations of AB in marine animals is still not completely understood. One suggestion is that it might work as an osmolyte like GB does. Accumulation experiments with different ratios of AB and GB have shown that more AB is accumulated when the GB concentration is low [25] and that *E. coli* accumulates AB with the transporters

of GB [26]. Furthermore Fujihara et al. have shown a negative correlation between the concentrations of AB and GB in animal tissues [27] and Clowes and Francesconi have shown, that the uptake and elimination of AB by the mussel *Mytilus edulis* depends on the salinity of the seawater. The higher the salinity has been in the water, the higher concentration of AB could be found in the mussel [28].

By consuming marine animals human can ingest high amounts of AB. Even these high amounts are of no known toxicity (see Table 2). One reason for this might be the fast excretion of AB via the urine. Studies in which the arsenic concentration after fish consumption was measured, showed that AB was excreted with a half-life of ~7-11 hours [29, 30]. Furthermore the study showed that the way of AB in the body is still not understood. A second half-life of 76 hours [30], respectively 63 hours [29], for the still retained AB, showed a longer retention in the human blood than supposed. Most people think that AB is excreted without undergoing any reaction in the human body. The observation of a second, much longer half-life doubts this thought. A study, published in 1983 by Vather et al. supports the opinion, that AB is excreted without being metabolized. In this study ^{73}As-lablled AB was orally ingested in mice, rats and rabbits. As a result ^{73}As-lablled AB was the only observed ^{73}As-lablled arsenic species in the urine and tissue extracts of those animals [31]. Obviously this study was not able to answer the question of the second half-life either, so more studies on the behaviour of AB in the human body are necessary.

2.1.4 Arsenic in the environment

Arsenic can occur in really high concentrations in some soils [32]. The major differences are geographically determined. In west Bengal (India) for example the arsenic concentration in soils ranges from 10-196 mg/kg [33]. In Germany, in the surrounding of Berlin, only 2.5-4.6 mg/kg could be observed [32].

The WHO recommend value for arsenic in drinking water is < 10 µg/L. Typical concentrations of arsenic in unpolluted ground water are below 1 µg/L [34], in polluted areas values up to 5300 µg/L could be found [35]. Consequently many people in these polluted areas suffer from arsenic poisoning. Those high amounts of arsenic in these areas are mainly caused by the soil. The influence of anthropogenic pollution is small.

2.2 Betaine-Homocysteine-Methyltransferase (BHMT)

2.2.1 Introduction in BHMT

Betaine-homocysteine-methyltranferase (BHMT) is a zinc dependent enzyme, highly expressed in the liver and the kidneys. It catalyses the transfer of a methyl group from GB, or only betaine, to homocysteine to generate methionine and dimethylglycine (see Figure 1).

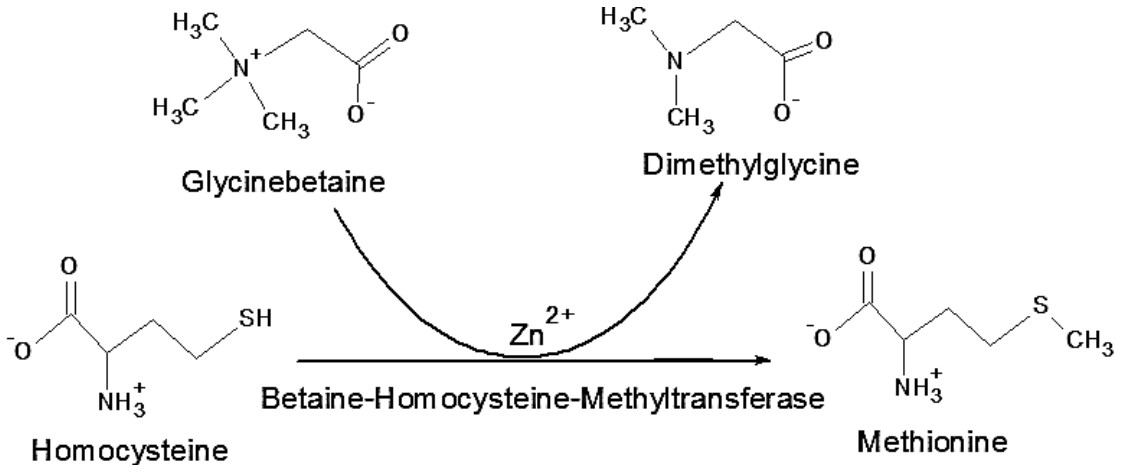


Figure 1 Reaction of betaine-homocysteine-methyltransferase (BHMT) [36]

Glycinebetaine

Glycinebetaine is an essential osmolyte and methyl donor [37]. It is named after the sugar beet (*Beta vulgaris*) from which it was first isolated in the 1860s by Scheibler [38]. It can be taken up with the diet or produced by the oxidation of dietary choline. Plasma betaine ranges from 20-60 µmol/L in women and 25-75 µmol/L in men [39]. Betaine is used in animal feed to increase the muscle mass and decrease the fat content. This effect has been observed for pigs and poultry [40-42] but not for humans [43]. In human, betaine is used against hyperhomo-cysteinemia. Furthermore there have been studies about the positive effects of betaine on the risk of cardiovascular disease [44], the risk of breast cancer [45] and autism [46]. Most of these studies are not well proven, but there is a lot of research on the positive effects of betaine for the human body.

Dimethylglycine

Dimethylglycine degrades to sarcosine and further to glycine. Plasma dimethylglycine levels are typically < 10 µmol/L.

Homocysteine

Usually men have higher plasma homocysteine levels than women and it increases with age. 10.8 µmol/L (for men) and 9.1 µmol/L (for women) total plasma homocysteine could be observed in 40 to 42 years old people. At the age of 65 to 67 years the levels raise up to 12.3 µmol/L for men and 11.0 µmol/L for women. The plasma homocysteine level increases with the number of smoked cigarettes and correlates positively with total cholesterol levels and blood pressure as well [47]. Plasma homocysteine levels >15 µmol/L are called hyperhomocysteinemia which increases the risk of thrombosis and cardiovascular diseases [48].

Methionine

Methionine is an essential amino acid which is converted to S-adenosylmethionine (SAM), the major biological methyl group donor and is involved in protein synthesis [49].

2.2.2 Methionine metabolism

Generating methionine from homocysteine with BHMT is only one reaction of homocysteine. As shown in Figure 2 methionine can be formed from homocysteine by methyltetrahydrofolate-homocysteine-methyltransferase (MTR) as well (reaction 8). This enzyme needs methyl-cobalamin as cofactor. A third reaction of homocysteine leads to an exit of the methionine-homocysteine-cycle (reaction 4-6). In this reaction homocysteine is irreversible converted to cystathione with cystathione-β-synthase and serine. This is cleaved to α-ketobutyrate and cysteine by γ-cystathionase. Further metabolism of cysteine leads to inorganic sulfate. Of course homocysteine can re-synthesis S-adenosyl-homocysteine by reversing the adenosyl-homocysteinase reaction (reaction 3) [36, 49].

The activity of the different reactions is affected by nutrition. BHMT activity increases with protein and methionine rich diet and MTR activity increases when methionine is needed. This leads to the assumption that BHMT is primarily used to metabolize excess of homocysteine or betaine [50]. A big difference of these two re-methylation pathways of homocysteine is their contribution in the body. BHMT is mainly found in livers [50]. Additionally small contributions have been observed in kidneys of Rhesus monkeys [51], pigs [52] and human, post-mortem [53]. In contrast MTR is found in nearly all tissues [50].

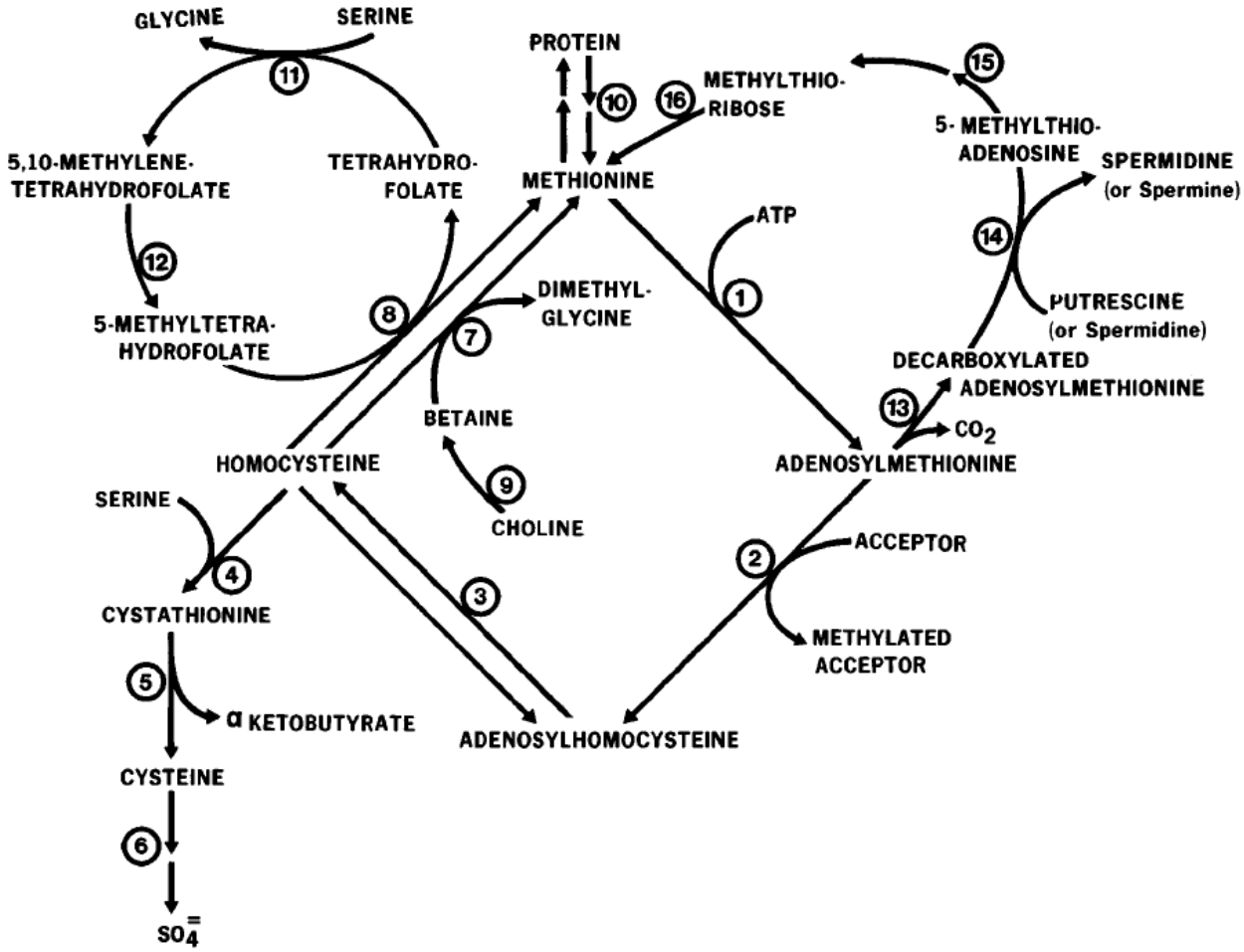


Figure 2 Methionine metabolism in mammals, adapted from Finkelstein and Martin [36]; 1 methionine adenosyl-transferase, 2 adenosylmethionine dependent transmethylations, 3 adenosyl-homocysteinase, 4 cystathione β-synthase, 5 γ-cystathionase, 6 multiple reactions leading from cysteine to sulfate, 7 betaine-homocysteine-methyltransferase (BHMT), 8 methyltetrahydrofolate-homocysteinemethyl-transferase (MTR), 9 choline dehydrogenase + betaine-aldehyde dehydrogenase, 10 equilibrium between protein methionine and free methionine, 11 serine hydroxymethylase, 12 methylenetetrahydrofolate reductase, 13 adenosylmethionine decarboxylase, 14 spermidine synthase, 15 methylthioadenosine phosphorylase, 16 converstion of methylthioribose 1-phosphate to methionine

2.2.3 Structure and reaction mechanism of BHMT

BHMT consists of a dimer of dimers resulting in four identical subunits, each folded into a $(\beta/\alpha)_8$ barrel (see Figure 3) [54]. The active site is built up by distortion of the β-strands of the barrel [55]. BHMT uses zinc to activate the thiol-group of homocysteine for nucleophilic attack [56]. The reaction of BHMT conforms to a bi-bi-mechanism whereas homocysteine is the first substrate bound to the enzyme and dimethylglycine is the first product released. The supposed transition state and the assumed bi-substrate are shown in Figure 4. The activating zinc atom is tetrahedral coordinated, at the active site of BHMT, by 3 thiolate

anions, from 3 cysteine molecules, and the hydroxyl group of a tyrosine. When homocysteine binds to BHMT, it replaces the tyrosine and this leads to conformational changes, which create the binding site of betaine in the enzyme [55, 57]. The reaction of BHMT is inhibited by its products (dimethylglycine and methionine) as well as L-cysteine and L-cystine.

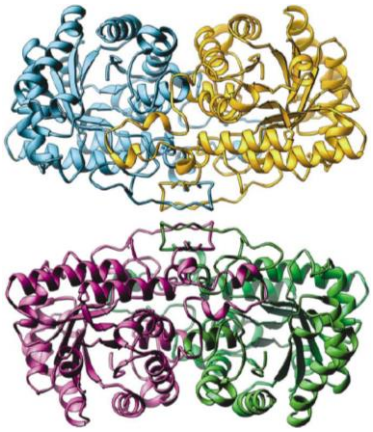


Figure 3 BHMT tetramer adapted from Evans et al. [54]; colouring distinguishes the four subunits of the tetramer

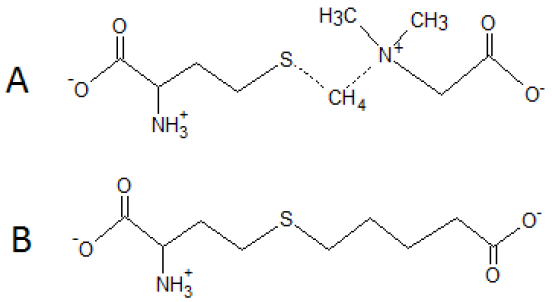


Figure 4 Transition state of BHMT; homocysteine + betaine (A) and the bi-substrate S-(δ-carboxylbutyl)-L-homocysteine (B) [58]

2.3 Urine Analysis

2.3.1 Introduction in urine analysis

Urine analysis (uroscopy) has a long history in medical analysis. The composition of urine provides important information about the health status of a patient because some compounds should be present in a certain concentration range and others should not be present at all. A big advantage of urine analysis is the simple, non-invasive sample collection. Although it is really easy for the patient, it is not that easy for the doctor. Before taking urine samples it is important to know what parameters should be measured, because there are different ways of sample collection dependent on the values of interest.

2.3.2 Different types of urine collection

- Random urine samples

  These urine samples can be collected at any time; therefore, they are the easiest available urine samples and most commonly used ones. They are used for urinalysis and microscopic analysis, although they are not the samples of choice for both tests. The problem with the random urine samples is that they may not be representative and furthermore to low concentrated urine can lead to wrong interpretations and give an incorrect view of the patient's health status.

- First morning urine samples

  The commonly used urine is the first urine in the morning. To get reliable results the patient had to empty his bladder before going to sleep. The received urine can also be called 8-hours urine because this is the average duration of a night and therefore, the whole urine over 8 hours is collected. If the patient needs to urinate during the night he has to pool this urine too, to get a true 8-hours urine sample. This is the favoured urine for urinalysis and microscopic analysis because it is more concentrated than the randomly collected one.

- Midstream clean catch urine samples

  For this sample collection the patient first has to clean his urethral area. Then he should drain the first few millilitres of urine in the toilet and collect only the urine midstream. This type of urine collection can be done at any time, like the random urine samples, and has the advantage to suffer from fewer cellular and microbial contaminations. Therefore, the midstream clean catch urine is the favoured one for culture and sensitivity testing.

- Time collected urine samples

  Before starting this type of urine collection, the bladder of the patient must be emptied. Then the complete urine over a specific time period, usually 8 or 24 hours, is collected and pooled together. At the end of the sampling period the last sample has to be collected. This pooled urine is used for the measurement of concentrations of creatinine, glucose, sodium, potassium and many other compounds. To receive reliable results a proper timing is very important.

- Catheter collected urine samples

  In this method a catheter is inserted into the bladder of the patient to collect the urine. This is only done if the patient is bedridden or cannot urinate himself.

- Suprapubic aspiration urine samples

  In this method the urine is collected with a needle directly from the bladder. This is used if sterile urine samples are required or if the patient is bedridden, cannot urinate himself and cannot be catheterized either [59].

2.3.3 Urine analysis – Some problems

A big disadvantage of urine analysis is its composition. Urine consists of many different compounds and their concentrations can be easily influenced by nutrition and the drinking behaviour and not only by the health status. Furthermore some compounds can influence the measurement of others negatively. The major compounds of urine are listed below in their approximate amount in urine per day.

Organic substances:

- 20 g urea
- 2 g amino acids
- 1.2 g creatine
- 0.5 g citric acid
- 0.5 g uric acid
- 0.5 g NH_3
- 0.06 g proteins

Inorganic, cationic substances:

- 5.9 g Na^+
- 2.7 g K^+
- 0.8 g NH_4^+
- 0.5 g Ca^{2+}
- 0.4 g Mg^{2+}

Inorganic, anionic substances:

- 8.9 g Cl^-
- 4.1 g PO_4^{3-}
- 2.4 g SO_4^{2-}

Volatiles:

- Alkyl furans
- Ketones
- Lactones
- Pyrrole
- Allyl isothiocyanate
- Dimethyl sulfone

[60]

Dependent on the amount of water the patient has drunk the matrix and the compounds observed are more or less concentrated. Another important thing is that many substances from our body are excreted via the urine; hence its composition depends on our food intake as well. A good example is arsenic in seafood. High amounts of arsenic, especially arsenobetaine (AB), an organic, nontoxic arsenic species, are ingested when eating sea fish. A few hours later, high concentrations of arsenic can be found in the human urine without any signs of poisoning. This is because AB is excreted quite fast.

2.3.4 Normalisation of urine samples

One way to enable more accurate quantifications in urine samples is the normalisation of the urine. This can be done in different ways: One method is based on the urinary excretion rate (UER), another on the concentration of creatinine in the sample and a third one normalises the samples according to their specific gravity. All three methods try to normalise the urine, considering its water content, to make results comparable [61, 62].

The urinary excretion rate is the total mass of analyte in a total urine void. The method makes the assumption, that the entire bladder is emptied with each void. The UER can be calculated with Equation 1.

$$UER = \frac{c_u \cdot V_u}{(t_c - t_l)} \quad (1)$$

The concentration of the analyte (c_u) is multiplied with the total volume of the void (V_u) and divided by the duration of time the void was accumulated in the bladder (collection time t_c minus the time of the last urination t_l). To enable this calculation, the volume of the urine and the accurate time of the urination must be documented. This can be quite complicated when working with children or dealing with lots of samples. Therefore, other methods are commonly used [63].

When normalising the urine according to its creatinine concentration the concentrations of the analytes are reported as ratio to the creatinine concentration. Creatinine is a by-product of the muscle metabolism and hence can suffer from small day-to-day variations. Unfortunately the concentration of creatinine in the urine can depend on the diet [64], the age, the sex, the body mass index (BMI) [61] and some more factors as well. Therefore, this commonly used method is not the method of choice for some specific applications.

Urine specific gravity (USG) is the ratio between the density of the urine and the density of water. After measuring the USG the analyte concentrations can be corrected with Equation 2.

$$c_{corrected} = c_{raw} \cdot \frac{USG_{reference} - 1}{USG_{experimental} - 1} \quad (2)$$

$C_{corrected}$ is the adjusted and c_{raw} the not adjusted analyte concentration. $USG_{reference}$ is the mean USG from an appropriate population and $USG_{experimental}$ is the measured USG. For sure the USG can be influenced and adulterated as well. One reason for a wrong normalisation with USG would be if there are lots of heavy molecules, like proteins or glucose, in the urine. Such molecules can be easily analysed with routine urinalysis and hence the USG value can be corrected more easily than the creatinine concentration [62].

2.4 Solid Phase Extraction

Solid phase extraction (SPE) is an important sample preparation technique and can be described as a step-wise chromatography, extracting one or more compounds from a liquid phase (sample) onto a solid stationary phase. It uses the different affinities of compounds from the liquid sample with the solid sorbent material. The sample is passed through a cartridge with sorbent material resulting in retention of the analyte or the matrix compounds on the stationary phase. If the analyte is not retained, the fraction is collected. More commonly the analyte retains on the cartridge and has to be eluted afterwards. This way of solid phase extraction can be split in five parts which will be discussed below (see Figure 5).

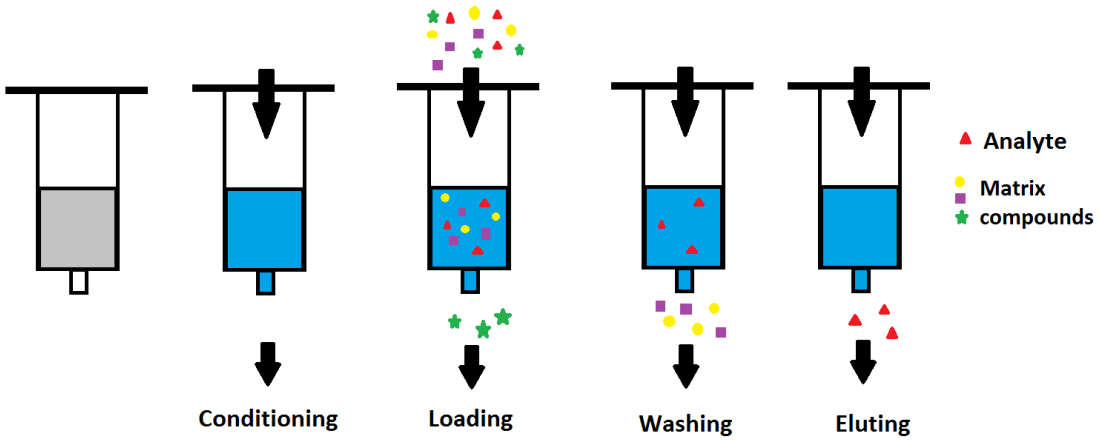


Figure 5 Scheme of solid phase extraction

Before starting the sample preparation, the SPE cartridge with a proper sorbent material and an adequate size has to be chosen. Like in chromatography there are normal-phase, reversed-phase and ion-exchange sorbent materials. More information about stationary phases can be found in chapter *2.5.1 Setup and mechanism of an HPLC*. The needed bed mass depends on the amount of analyte that should be adsorbed. By using reversed-phase or normal-phase the mass of the extracted compound should not exceed 5 % of the mass of the packing material. By using ion-exchange materials the exchange capacity should always be determined.

The next step is the conditioning step. To condition the sorbent material, approximately the threefold volume of the cartridge, of the conditioning agent, is passed through it dropwise, using either positive pressure or vacuum. This is done to wet and activate the functional groups of the sorbent material. Therefore, the choice of conditioning solvent depends on the sorbent material and the sample. Starting from now, a thin film of solvent should always remain on the sorbent to prevent drying of the stationary phase.

After the preparation the sample can be added and passed through the cartridge dropwise. The flow rate can affect the retention of some compounds and should be unchanged.

In the next step - the washing step - an adequate solvent is passed through the cartridge to elute as much matrix compounds as possible, but not the analyte. The choice of a proper solvent depends on the sorbent material, the analyte and the impurities. Frequently the solution in which the sample was dissolved is used. Usually not more than one cartridge volume of the washing solvent is needed.

In the last step the analyte is eluted from the sorbent material. Therefore, a solution that removes the analyte from the cartridge, but leaves behind any matrix compounds, not eluted in the washing step, is needed. The choice of the proper solvent depends again on the sorbent material, the analyte and the impurities. The collected eluting fractions can then be analysed [65, 66].

2.5 High Performance Liquid Chromatography

High performance liquid chromatography (HPLC) is a powerful separation technique for many different compounds and applications. The liquid sample is transported with a mobile phase through a stationary phase and the different compounds of the sample interact with the stationary phase differently, resulting in a separation.

2.5.1 Setup and mechanism of an HPLC

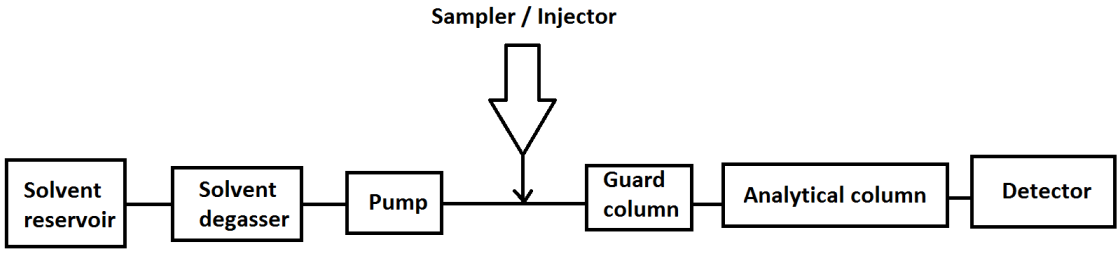


Figure 6 Scheme of an HPLC system

First a mobile phase is pumped through a solvent degasser and then a small amount of sample (typically 1-20 µL when using a column with 2 to 4 mm inner diameter) is added. The mobile phase, loaded with the sample, passes through a guard column before passing the analytical column. The guard column protects the analytical column from complex matrices and its necessity depends on the sample. Usually both columns consist of the same

stationary phase which is built up of small particles (~2-10 µm) of silica or organic polymers modified with functional groups. In the analytical column the interaction of the analyte with the functional groups of the stationary phase takes place and the sample compounds get separated. The used functional groups depend on the analyte and depending on them different separation techniques can be discussed.

Reversed-phase chromatography is the most common separation technique. The particles of the stationary phase are modified with long, nonpolar alkyl chains, typically C18 and C8. Common mobile phases are polar solvents like water, methanol or acetonitrile. The separation takes place because nonpolar compounds are interacting stronger and therefore, retain longer on the column than polar ones.

Especially for charged analytes ion-exchange chromatography is used quite often. The surface of the stationary phase is modified with charged groups. Anion-exchange columns (used to separate anions) contain a stationary phase modified with positively charged groups, like amines and quaternary ammonium groups. Cation-exchange columns (used to separate cations) consist of a stationary phase modified with negatively charged molecules, like sulfonic acids or carboxylic acids. The mobile phase is commonly an aqueous buffer containing counter ions, which are competing with the analyte ions for free position to interact with the stationary phase.

Behind the column the separated analytes reach the detector. There are many different detectors possible and the detector of choice depends on the analyte and the information needed. Often unselective detectors like refractive index, spectrophotometric or conductivity detectors are used. Furthermore, a mass spectrometer can provide important information.

2.6 Mass Spectrometry

Receiving a mass spectrum of a sample can give important information about its purity and composition. Because of this, mass spectrometry is an important tool in many fields of analytical chemistry. There are many different types of mass spectrometer and most of them consist of the four parts shown in Figure 7.

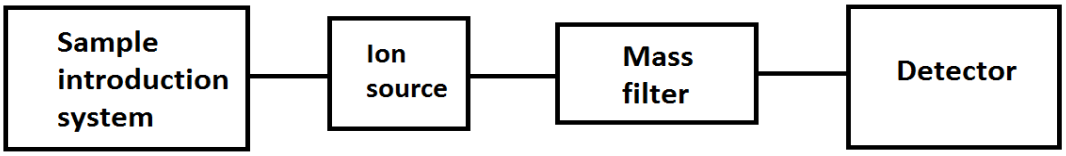


Figure 7 Scheme of a common mass spectrometer

For mass spectrometry the sample can be liquid, introduced with an autosampler or arising from an HPLC, gaseous, transported by a stream of argon or arising from a gas chromatography system or solid, introduced by laser ablation. Depending on the sample and ion source the sample has to be prepared and introduced in different ways. For example a liquid sample has to be sprayed to generate small droplets which can enter the ion source.

The generation of ions can be done in many different ways, depending on the information needed. The ionization methods can be grouped in soft and hard ones, depending on the type of ions generated. Soft ionization methods generate molecule ions. This means that the whole molecules of the sample get ionized without breaking or at least only little fragmentation. Fragmentation means that the molecule breaks at specific bonds and then these parts get ionized. Some examples for soft ionization methods are matrix assisted laser desorption ionization (MALDI), atmospheric pressure chemical ionization (APCI), atmospheric pressure photoionization (APPI) and electrospray ionization (ESI), which is the softest ionization technique and will be discussed in more detail in chapter *2.6.2 Electrospray mass spectrometry*. The harder the ionization technique is the more fragment ions are generated. These fragments can give important information for structure determination. Hard ionization techniques generate no molecular ions but fragment and element ions. A common rather hard ionization technique is electron impact (EI). The hardest ionization technique, which generates only element ions, is inductively coupled plasma ionisation (ICP) which will be discussed in more detail in chapter *2.6.1 Inductively coupled plasma mass spectrometry*.

After ionizing the sample, the ions have to be separated according to their mass to charge ratio (m/z). One of the most common mass filters is a quadrupole; explained in more detail in chapter *2.6.1 Inductively coupled plasma mass spectrometry.* Time of flight or sector field mass filters are employed to separate ions as well.

After the separation of the ions they have to be detected. This is commonly done with an electron multiplier which transforms the arriving ions to an electric signal which can be monitored on the computer. The signal intensity is directly proportional to the amount of ions which could pass the mass filter and this is directly proportional to the concentration of this ion.

2.6.1 Inductively coupled plasma mass spectrometry

Inductively coupled plasma mass spectrometry (ICPMS) is a powerful analytical technique. “Simultaneous” detection of nearly all elements of the periodic table, isotopic information, a really low limit of detection (< 1 ng/L, depending on the element) and a high dynamic range (10^9 to 10^{10} depending on the instrument) makes it to the technique of choice for many applications, like environmental, geological, food, clinical, industrial or pharmaceutical analysis. Disadvantages of an ICPMS are of course its high costs and its complexity which requires well educated operators.

Setup of an ICPMS

Solid, liquid and gaseous samples can be analysed with ICPMS although commonly liquids are introduced. These samples can be introduced with an autosampler or can arise for example from an HPLC. A nebulizer generates an aerosol. This aerosol is transported into the spray chamber, in which big droplets (>10 µm) are removed and only small ones are further transported to the torch (see Figure 8).

Commonly the torch consists of three concentric quartz tubes. The plasma gas flows between the outer tube and the middle tube. It builds up the plasma and cools the outer tube. The auxiliary gas flows between the middle tube and the innermost. The sample is introduced with the carrier gas only in the innermost tube. All in all about 15 to 20 L gas flow through the torch per minute. Commonly all three gases are argon, because it is cheap, inert and its ionisation energy is 15.75 eV. This ionisation energy leads to singly charged ions for most of the elements. Other gases can be introduced between the spray chamber and the torch which are called optional gas and will be discussed later. At the end of the torch a water-cooled copper coil is mounted, providing the magnetic field which is needed for the plasma formation. The plasma is generated by a high-voltage spark producing free electrons, accelerating in the magnetic field. This leads to collisions and ionization of the argon gas which forms the plasma, reaching around 6000-10000 K.

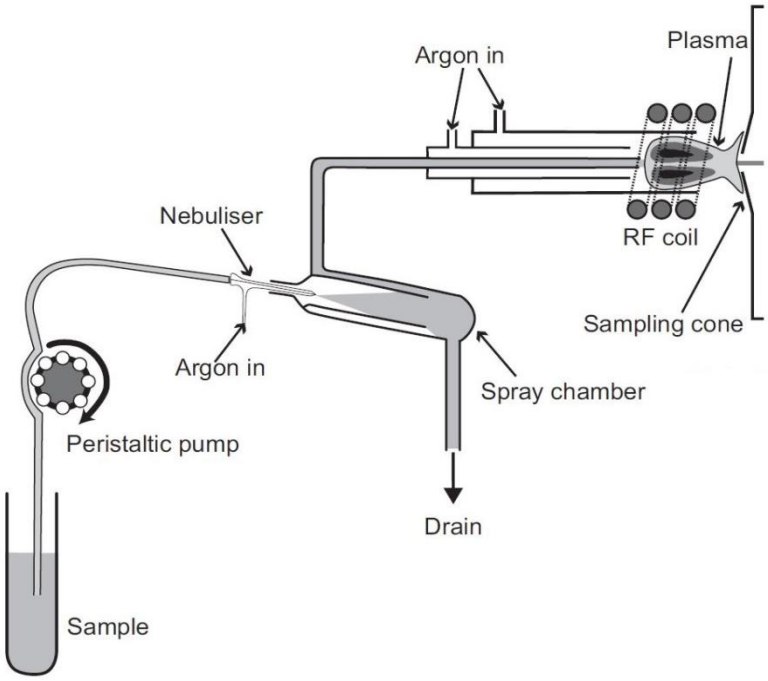


Figure 8 Scheme of the sample introduction into an ICPMS, adapted from Linge and Jarvis [67]

In the plasma the small sample droplets are dried, evaporated, atomized and finally ionized. This part of the ICPMS is still under atmospheric pressure. In the next step the ions enter a high vacuum through the interface (see Figure 9). This consists of two cones, a sample and a skimmer cone, which are two round plates of Ni, Cu or Pt. The sample cone is directly in contact with the plasma and has a small opening (~1 mm). After passing through the sample cone the ions are in a low vacuum region and have to pass through the skimmer cone (opening ~0.4-0.7 mm) to enter the high vacuum region in which the ion lenses focus the ion beam and remove neutral particles.

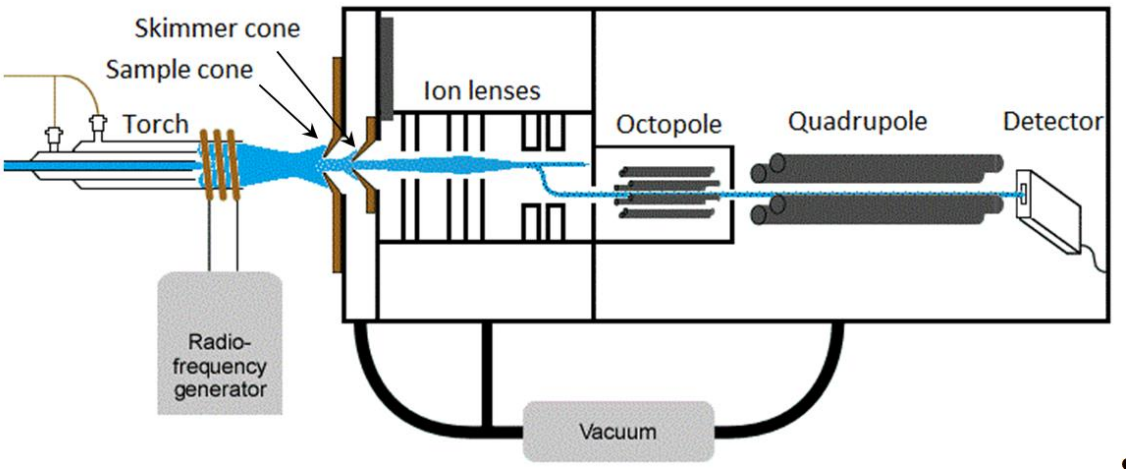


Figure 9 Scheme of an ICPMS, adapted from Dunnivant and Ginsbach [68]

The next part of the ICPMS is the collision or reaction cell which can be used when needed. In the Agilent systems it is an octopole which means that it consists of eight rods with applied current to keep the ions on track. Depending on the operating mode a collision or a reaction gas is introduced with about 4 mL/min. These gases are used to reduce interferences from the ion beam.

In the collision mode for example He, which is inert, is used. He atoms are colliding with the ions and there are two mechanisms to reduce polyatomic interferences with this method. The first one is the energy discrimination; the collision reduces the kinetic energy of the ions. Polyatomic interferences (ions consisting of more than one atom) have a larger cross section than analyte ions and are colliding more often. Their kinetic energy is reduced and applying an energy barrier between octopole and mass filter exclude them. The second mechanism is the collision induced dissociation; the collision of polyatomic interferences with a He atom can lead to the dissociation of the interference. The collision mode is used when polyatomic interferences play an important role. An example would be the analysis of arsenic. $^{75}As^{+}$ and $^{40}Ar^{35}Cl^{+}$ both have m/z = 75. The use of the collision cell can help reducing $ArCl^{+}$ and generating more accurate results for arsenic.

In the reaction mode for example H_2 is used. H_2 can undergo reactions with the interferences or with the analyte of interest. Therefore, a good knowledge of the matrix is necessary.

After passing the collision/reaction cell the ions enter the mass filter. The most common mass filter in ICPMS is a quadrupole. It consists of four rods, arranged in a square. A direct current is applied and overlaid with an alternating current. Together they generate a field in which only ions with a specific mass to charge ratio (m/z) can oscillate through and enter the detector. By shifting the current settings different m/z can pass through the quadrupole. This means that only one m/z after another one can enter the detector. The detector transforms the arriving ions to an electric signal which can be monitored on the computer. The signal intensity is directly proportional to the amount of ions which could pass the quadrupole with the specific setting and this is directly proportional to the concentration of this ion [67, 69].

Optional gas & carbon enhancement effect

In the used ICPMS settings 16 % optional gas (1 % CO_2 in Ar (v/v)) were added between the spray chamber and the torch to benefit from the carbon enhancement effect. This is the signal enhancement of some elements like As and Se, up to a factor of 5, by increasing the carbon content in the plasma. First experiments concerning the carbon enhancement effect were performed by adding organic solvents to the samples. Later the organic solvents were

introduced directly into the spray chamber. Finally the most efficient and easiest way is to add the carbon via a gas flow, like we did in our experiments. Another benefit of adding high carbon content is the minimisation of matrix effects of some samples. If some samples have higher carbon content than others, or than the calibration standards have, the measured concentrations might not be correct. High external added carbon content can minimize this differences and lead to better limits of detection. Till now, the mechanism of the carbon enhancement effect is not well understood, but many people try to explain it with a charge transfer effect [70-72].

2.6.2 Electrospray mass spectrometry

Electrospray mass spectrometry (ESMS) is one of the most important mass spectrometric techniques to receive molecular mass information. It is one of the softest ionization techniques leading to ions of the whole molecules. This makes it to an important tool for biomedical and pharmaceutical research, especially for impurity analysis, protein characterization, metabolite identifications and pharmacokinetics.

A big problem of ESI is its high susceptibility to matrix effects and signal suppression which will be discussed in more detail later.

Setup of an ESMS

Like most mass spectrometers an ESMS consist of a sample introduction system, an ion source, a mass filter and a detector. As shown in Figure 10 only the ion source is different to the ICPMS. This is the reason why only this part of the ESMS will be discussed in this chapter.

Figure 10 shows an ESMS connected with an HPLC system. The arriving sample is passing through a needle on which a high positive or negative potential (depending on the measuring mode) is applied. Applying positive potential on the needle leads to positively charged droplets, applying a negative potential leads to negative ones. Further explanation of the mechanism of ESI will be discussed only in positive mode: When the sample reaches the high electric field at the needle tip, the anionic species are attached to it and the positive ones accumulate on the meniscus surface building up a Taylor cone (see Figure 11).

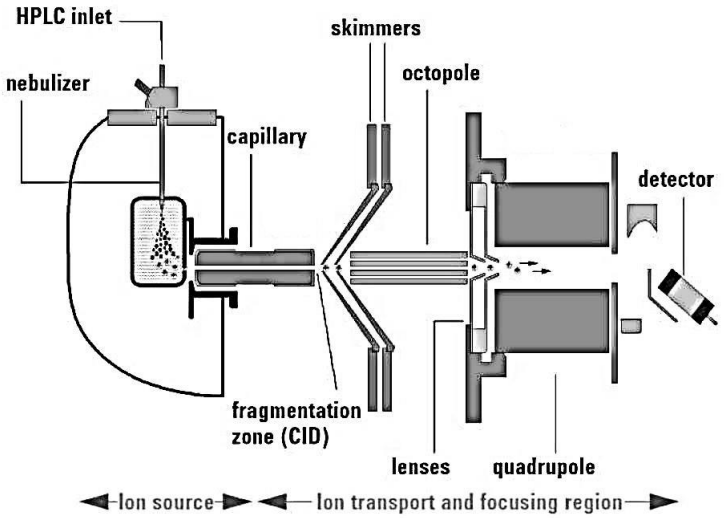


Figure 10 Scheme of an ESMS adapted from Agilent Technologies [73]

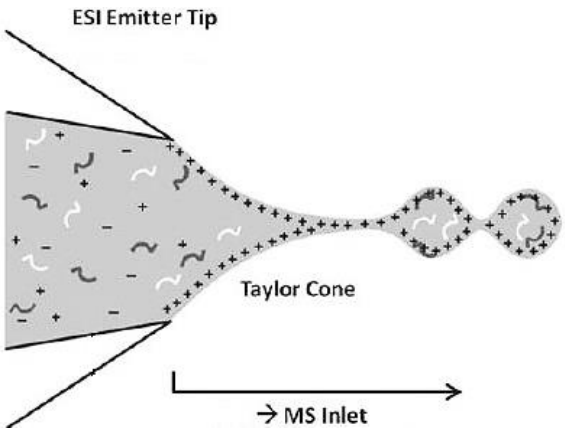


Figure 11 Scheme of the formation of a Taylor cone in ESMS adapted from Hawkridge [74]

More and more positive charges accumulate and increase the potential till the positive charge overcomes the surface tension and small droplets are formed at the tip of the Taylor cone. These droplets build up an aerosol spray. The spraying efficiency can be increased with a nebulizer gas flow passing the end of the needle. Before entering the mass spectrometer the solvent of the droplets gets evaporated. Thereby the droplets get smaller and smaller and the density of the positive charges on the droplet surface increases until the Rayleigh stability limit is reached. This means that the charge repulsion overcomes the surface tension which leads to a breakdown of the droplets until only gas phase ions are left (see Figure 12). Because of this soft ionization mechanism nonpolar molecules like alkanes are not ionized at all.

The ions move to the counter capillary. Depending on the instrument there are different arrangements. Figure 10 shows the common arrangement in which the capillary is orthogonal to the needle and only ions can enter it. In Figure 12 the capillary is in front of the

needle. In this arrangement a sheath gas is needed to remove small neutral particles [75, 76]. After entering the capillary the ions pass the same parts of a spectrometer like in ICPMS.

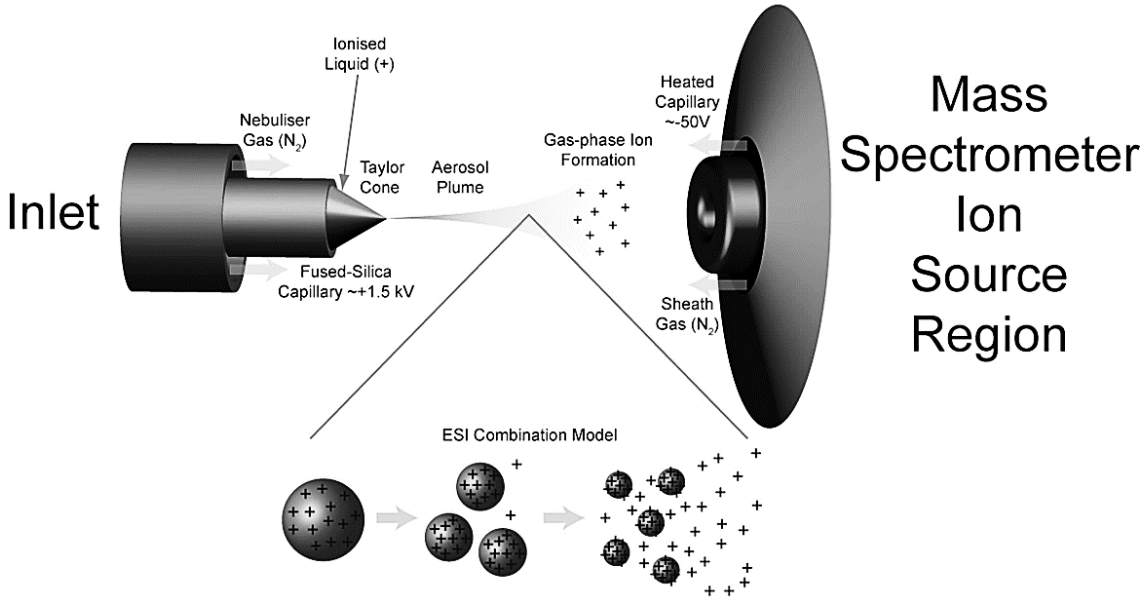


Figure 12 Scheme of an electrospray ion source adapted from Lamond Lab [77]

Signal suppression; a problem in ESMS

There have been many studies investigating the response observed in ESMS under different conditions, reasoning that the signal intensity is affected by many factors beside the analyte concentration. Some of these factors are listed in Table 5. Another problem in ESMS is that the influence of these factors often depends on the analyte concentration, which complicates quantifications even more.

Table 5 Factors affecting analyte response in ESMS [78]

System variables	Method variables	Compound variables
Electric field	Flow rate	Surface activity
ES-capillary diameter	Electrolyte concentration	Proton affinity
ES-capillary voltage	pH	pK_a
Distance to counter electrode	Solvent properties (e.g. boiling point or surface tension)	Solvation energy
Heat capacity of ambient gas		
Solvent saturation level of ambient		

For example a smaller capillary diameter, used in nano-ESMS, can lead to better results. The smaller capillary diameter leads to smaller initial droplets, higher initial surface charge and faster droplet decomposition to ions, resulting in a better response [79].

Matrix compounds can lead to matrix effects by influencing the response of an analyte. In the time, when matrix compounds enter the ES source, they change the solvent properties. Non-volatile salts, for example, increase the boiling point and the osmolarity of the solvent, affecting the solvent evaporation, the droplet formation and finally the ionization of the analyte, leading to signal suppression [78]. Matrix compounds can change the conductivity and surface tension of the solvent as well. Salts and charged species increase the conductivity of the liquid and a higher conductivity leads to a worse behaviour of the spray and furthermore a worse ionization [80].

These problems occur if matrix compounds and analyte molecules enter the needle of the ES-source simultaneously. This can be caused by injecting a sample directly without separating its different compounds, by co-elution in a HPLC system or by mobile phases with higher gas-phase proton affinity than the analyte. Unfortunately not everything can be ionized at the same time; hence the analyte and the co-eluting compounds compete against each other [81]. This means that the concentration of ions from the analyte will decrease by increasing the amount of other ionized compounds and this leads to worse limit of detection and can end in a total suppression of the analyte signal [82].

Volatile ion-pairing reagents are often used in LC-ESMS but many of them lead to signal suppression. A study comparing the signal intensities of 8 amines has shown that the signal intensities were only 45-60 % when using trifluoroacetic acid (TFA) and 15-30 % when using perfluoroheptanoic acid or heptafluorobutanoic acid instead of formic acid, added to an ammonium formate buffer [83].

Beside signal suppression, there can be positive matrix effects too, leading to signal enhancement. It has been reported, that the addition of high levels of ammonium acetate can counteract the signal suppression caused by NaCl. A 3 fold improvement in the signal to noise ratio (S/N), has been observed, by adding 8 M ammonium acetate to an aqueous solution with cytochrome c without NaCl. Adding 7 M ammonium acetate to a 10^{-5} M cytochrome c and ubiquitin solution with $2 \cdot 10^{-2}$ M NaCl the S/N was improved by factors of 7 and 11 [84].

Another example for counteracting signal suppression has been reported by Apffel et al. in 1995. They have shown, that the signal suppression occurring from TFA, used in the separation of peptides, can be counteracted by adding a mixture of 75 % propionic acid and 25 % 2-propanol 1:2 with the mobile phase (all ratios v/v), between the HPLC and the ESMS.

This addition led to a 10-100 fold improvement of S/N. It is thought, that TFA lead to signal suppression because of its influence on the surface tension and the formation of ion-pairs between TFA anions and analyte ions. The mixture of organic solvents drives TFA to neutral TFA which evaporates from the droplets before the formation of gas-phase analyte ions [85].

A further example for the positive effects of post column addition of organic solvent has been reported by Yamaguchi et al. in 1999. They added 50 µL/min 2-(2-methoxyethoxy)ethanol post column to 100 µL/min 50 mM ammonium acetate buffer (pH 4.4, adjusted with acetic acid) to counteract the signal suppressive effects of acetate anions leading to a 100 fold signal enhancement by measuring ibuprofen with negative ESMS [86].

Chang et al. reported 2002 that a fused-droplet electrospray ionization (FD-ESI) source leads to really high salt tolerance when measuring peptides and proteins. In this method the sample solution was ultrasonically nebulized to generate an aerosol which was led into a glass reaction chamber. Charged methanol droplets were generated continuously with the ES. The capillary of the ES was placed in the centre of the reaction chamber and when the sample aerosol entered the reaction chamber, it fused with the charged methanol droplets. The mass spectra of ESMS and FD-ESMS were similar. With FD-ESMS cytochrome c could be detected as good in 10 % NaCl or 2.5 % NaH_2PO_4 solutions as in solutions without these matrix compounds, which shows the really high salt tolerance of FD-ESMS [87].

To avoid influences of the matrix different methods are possible to separate the analyte from the matrix. Usually the disturbing compounds are removed from the solution before entering the ESMS source. HPLC, SPE and dialysis are commonly used methods, easily automated, to clean-up samples before ESMS.

When using chromatographic separation of the different compounds, the unknown retention times of the matrix compounds can be a further problem. The analyte might elute after a few minutes and the major part of the impurities too. But there is no certainty that no matrix compounds are left on the column. It can happen that some compounds retain on the column the whole run of a sample and start eluting several samples later by falsifying their results. Therefore, it is important to prove, if some compounds are still retained on the column by observing a really long run or using a gradient, when measuring high matrix samples directly with LC-ESMS.

3 Experimental

3.1 Instruments

3.1.1 ICPMS, ESMS and HPLC

Agilent Technologies (Waldbronn, Germany):

7500ce ICPMS, equipped with an ASX-500 autosampler (Cetac, Nebraska, USA), a MicroMist nebulizer and a Scott-type spray chamber
7700x ICPMS, equipped with an Integrated Autosampler, a MicroMist nebulizer and a Scott type spray chamber

6120 Quadrupole LC/MS, equipped with a degasser (1260 Infinity), a binary pump (1260 Infinity), a thermostated autosampler (1290 Infinity) and a thermostated column compartment (1290 Infinity)

1200 Series HPLC, equipped with a degasser, a quaternary pump, a thermostated autosampler and a thermostated column compartment
1260 Infinity HPLC, equipped with a degasser, a binary pump, a thermostated autosampler and a thermostated column compartment

3.1.2 HPLC columns

Agilent Technologies (Waldbronn, Germany):

Cation-exchange: Zorbax 300-SCX Analytical 150 x 4.6 mm, 5 µm, SN: USSD004438

Hamilton (Bonaduz, Switzerland):

Anion-exchange: PRP-X100, 150 x 4.6 mm, 5 µm, SN: 573
Guard column: PRP-X100, PEEK, 8 x 3 mm, 10 µm

3.1.3 Solid phase extraction

SPE-vacuum manifold: Visiprep™ DL SUPELCO (Bellefonte, United States)

SPE cartridge:

LiChrolut® SCX (40-63 µm) 500 mg, 3 mL PP Merck KGaA (Darmstadt, Germany)
Bond Elut Plexa PCX, 60 mg, 3 mL Agilent Technologies (Waldbronn, Germany)

3.1.4 Other instrumentes

Balances: Denver Instrument®, SI-234, (Goettingen, Germany)

Pipettes: Acura 825 autoclavable, 100-1000 µL and 10-100 µL, Socorex (Ecublens, Switzerland)

pH-meter:

Orion 5 Star, Thermo Scientific (Cambridgeshire, United Kingdom)
pH 1000 L, VWR (Radnor, Pennsylvania, USA)

Total Solids Refractometer: TS400, Serum Protein 6.54, Leica Microsystems Inc. (Buffalo, USA)

3.2 Materials

PP-tubes: Polypropylene, Cellstar®, graduated, conical bottom, blue screw cap, sterile, 15 and 50 mL, Greiner bio-one (Kremsmuenster, Austria)

PS-tubes: Polystyrene, GLKL, 12 mL Greiner bio-one (Kremsmuenster, Austria)

Filters: 25 mm, bright blue, membrane: Nylon (PA), 0.2 µm, Markus Bruckner Analysentechnik (Linz, Austria)

Syringes: Injekt®Solo, Single-use, 2-piece, 2 mL, B. Braun Melsungen AG (Melsungen, Germany)

Parafilm®: 4In.x125FT.Roll, PM-996, Benis Flexible Packaging (Neenah, USA)

HPLC-vials: Microvials PP, 0.7 mL with snap ring, VWR International (Vienna, Austria)

HPLC-caps: Snap ring cap, 11 mm transparent, soft version, red rubber/PTFE, 45° shore A, 1.0 mm, VWR International (Vienna, Austria)

Combur^{10}Test®: urine test strips, Roche Diagnostics (Rotkreuz, Switzerland)

3.3 Chemicals

3.3.1 Standards

CertiPUR® ICP multi-element standard solution VI, for ICP-MS (30 elements in dilute nitric acid), UN2031, Merck KGaA (Darmstadt, Germany)

Single-Element ICP-Standard-Solution Roti®Star, Carl Roth GmbH+Co.KG (Karlsruhe, Germany)

Ba: 1000 mg/L ± 0.2 % in 1 % HNO_3, Art.No.: 2400.1
Ca: 10 000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2503.1
Cd: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2405.1
Co: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2410.1
Cu: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2426.1
Fe: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2412.1
Ge: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 1419.1
In: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2423.1
K: 10 000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2519.1
Mg: 10 000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2524.1
Mo: 1000 mg/L ± 0.2 % in 4 % NH_3, Art.No.: 2438.1
Na: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2439.1
Ni: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2444.1
P: 10 000 mg/L ± 0.2 % in H_2O, Art.No.: 2534.1
Pb: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2403.1
Sb: 1000 mg/L ± 0.2 % in 20 % HCl, Art.No.: 2398.1
Se: 1000 mg/L ± 0.2 % in 2 % HNO_3, Art.No.: 2461.1
Zn: 10 000 mg/L ± 0.2 % in 3 % HNO_3, Art.No.: 2576.1

Peak Performance, Single Element Standard, CPI International (Santa Rosa, USA)

As: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-100031

B: 1000 ± 3 µg/mL in H_2O, P/N S4400-100074

Be: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-100051

Bi: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-100061

Cr: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000121

Hg: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000331

Li: 1000 ± 3 µg/mL in 1 % HNO_3, P/N S4400-1000291

Lu: 10 ± 0.05 µg/mL in 2 % HNO_3, P/N S4400-010031

Mn: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000321

Rb: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000451

S: 10 000 ± 30 µg/mL in H_2O, P/N S4400-10M544

Sr: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000531

Te: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000563

U: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000641

V: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000651

Y: 1000 ± 3 µg/mL in 2 % HNO_3, P/N S4400-1000671

Prepared in-house (Analytical Chemistry, University of Graz):

Arsenate (As(V)): 1000 mg/L, from $Na_2HAsO_4 \cdot 7\ H_2O$, Merck KGaA (Darmstadt, Germany)

Arsenite (As(III)): 1000 mg/L, from $NaAsO_2$, Merck KGaA (Darmstadt, Germany)

Arsenobetaine bromide (AB): 1000 mg/L, from trimethylarsine and bromoacetic acid according to McShane [88]

Deuterated arsenobetaine bromide (d^9AB): from trimethylarsine-d9 (using CD_3I) and bromoacetic acid [88, 89]

Dimethylarsinic acid (DMA): 1000 mg/L, from sodium dimethylarsinate, Fluka (Buchs, Switzerland)

Methylarsonic acid (MA): 1000 mg/L, from $NaAsO_2$ and MeI (Meyer reaction)

3.3.2 Certified reference materials

Standard Reference Material® 1640a, Trace Elements in Natural Water, NIST (Gaithersburg, USA)

Seronorm™ Trace Elements, Urine, BLANK, Lot FE1113**,** Sero AS (Billingstad, Norway)

3.3.3 Other chemicals

Ultrapure water: Resistance 18.2 MΩ·cm, Millipore (Bedford, USA)

Carl Roth GmbH+Co.KG (Karlsruhe, Germany)

Ammonium formate: ≥95 %
Calciumchlorid-Dihydrat: ≥99 %, p.a, ACS
Formic acid: ≥98 % p.a., Rotipuran®
Hydrochloric acid: ≥32 % p.a., Rotipuran®
Hydrogen peroxide: 30 % p.a. Rotipuran®, stabilized
Magnesiumchlorid-Hexahydrat: ≥99 %, p.a, ACS
Nitric acid: ≥65 %, p.a., Rotipuran®, subboiled in-house (Analytical Chemistry, University of Graz)
Potassium chloride: ≥99.5 %, p.a, ACS, ISO
Sodium chloride: ≥99.5 %, p.a., ACS, ISO

Merck KGaA (Darmstadt, Germany)

Ammonium sulfate: ≥99.5 %, pro analysis
Diammonium phosphate: ≥99 %, pro analysis
Pyridine: ≥99.5 %, pro analysis
Sodium oxalate: ≥99.95 %, pro analysis
Urea: ≥99.8 %, p.a
Uric acid: p.a

Fluka Chemie GmbH (Buchs, Switzerland)

Ammonium chloride: ≥99 %, purum p.a
L-Glutamine: ultra; ≥99.5 %, (NT)
L-Histidine: ≥99.0 %, (NT)
L-Lysine: purum; crystallized; ≥98 % (NT)

Messer (Gumpoldskirchen, Austria)

Argon: 5.0
Helium: 6.0
Optional gas: CO_2 (1 % v/v) 4.5 in Ar 5.0

3.4 Software

ChemStation Software for LC systems, Rev. B.04.05

MassHunter Workstation Software for ICPMS, G7201B, Version B.01.02

Calculations: Microsoft© Excel 2010 and 2013

Figures: Microsoft© Excel 2010 and 2013

Molecular structures drawn in: ChemSketch

Writing: Microsoft© Word 2010 and 2013

Fitting tool: developed in Fortran 90, Gauss-Newton-Marquardt-Method described in [90]

3.5 Urine Sampling

We collected 113 urine samples from 5 volunteers in 3.5 days. We started collecting the urine one day before ingesting deuterated arsenobetaine (d^9AB, 2 mg As) and continued the collection for another 2.5 days. The samples were named with a code. The first number was the number of the person (1-5), then two numbers for the date (22-25 because the samples were collected between the 22th and 25th of July 2014), and the last two numbers for the number of the urine sample of each day. For example 22304 was the fourth urine sample of person 2 on the 23th of July 2014.

Table 6 Volunteers of the urine study

Person	Sex	Age	Urine Samples	Urine [L]
1	male	48	17	8.4
2	female	26	28	6.3
3	female	23	29	16.6
4	female	17	23	3.3
5	male	27	17	5.6

During the study the volunteers urinated into a 1 L plastic bottle with a 100 mL scale. After recording the amount of urine and the time of the urination they put approximately 40 mL into a 50 mL PP tube and stored them at 4°C in the fridge. Every day in the morning and in the evening the samples were put into the freezer at -20°C until analysis.

Before freezing the sample, their specific gravity was measured with the total solids refractometer TS400. All first morning urine samples and some more samples, randomly distributed, were tested with a Combur^{10}Test® (Roche Diagnostics). These urine test stripes are a semiquantitative determination of specific gravity, pH, leukocytes, nitrite, protein, glucose, ketone bodies, urobilinogen, bilirubin, erythrocytes and hemoglobin. The results of all Combur^{10}Test® were unremarkable.

3.6 Sample Preparation

3.6.1 Filtration

Before injecting urine samples into the HPLC or the ICPMS the urine was filtered with a 0.2 µm Nylon syringe filter to get rid of solid particles.

3.6.2 Oxidation

For anion-exchange chromatography the filtered urine samples were oxidized with 10 % v/v of a 30 % H_2O_2 solution. The oxidation took about 2-3 hours at about 50°C.

The used certified reference materials were oxidized in the same way.

3.6.3 Sample preparation for the determination of total element concentrations

For the determination of total element concentration the urine samples were filtered with a 0.2 µm Nylon syringe filter and diluted 1 + 9 with ultrapure water and 10 % (v/v) nitric acid.

3.6.4 Sample preparation for speciation analysis

For cation-exchange the filtered samples and for anion-exchange the oxidized samples were used. Because of previous determination the total arsenic content was known before speciation analysis. This enabled a proper dilution (1 + 9 or even 1 + 19) for the samples high in arsenic, to match the calibration range.

3.7 Method development

3.7.1 SPE – a clean-up method for samples with complex matrix

To enable the measurement of traces of AB in the urine matrix with HPLC-ESMS we developed a clean-up method with SPE. Therefore, a Visiprep™ DL vacuum manifold was used. To optimize the clean-up we used two different cation exchange cartridges. The Bond Elut Plexa PCX polymer based cation-exchange cartridge and the LiChrolut® SCX, a silica based one. To obtain the best retention of AB we tried different ways to prepare the cartridge. For washing we always used ultrapure water and for eluting we used 100 mM aqueous ammonium formate.

For the method development we used human urine spiked with 80 µg/L DMA, 10 µg/L MA, 100 µg/L AB, 5 µg/L As(III) and 5 µg/L As(V), simulating an urine after fish consumption.

For better quantification the elution fractions were measured with HPLC-ICPMS instead of HPLC-ESMS. The results of SPE were compared to spiked urine without clean-up. This spiked urine sample was only passed through a syringe filter, to get rid of solid particles, and then directly injected with the autosampler of the HPLC.

Selection of an adequate cartridge material and conditioning method for SPE

Three different ways of cartridge conditioning were tested.

The condition of the SPE-cartridge in the first method was done by passing threefold cartridge volume with aqueous formic acid (pH 2.3) through it. Then 1 mL of urine was added (loading). Afterwards five times 1 mL of ultrapure water was used for washing. Finally 1 mL fractions of 100 mM ammonium formate were used to elute AB.

In the second method the threefold cartridge volume of ultrapure water and afterwards the same amount of 6 M HCl was passed though the cartridge and the pH of the last droplet was checked to be acidic. Then ultrapure water was passed though the cartridge until the pH of the droplets was neutral again. The loading, washing and eluting steps were the same in all three methods.

The third method was similar to the second one, apart from the HCl concentration. Because the silica based SPE-cartridge is not stable at low pH, 1 M HCl was used instead of 6 M.

The fractions of the SPE were measured with HPLC-ICPMS. The conditions can be found in Table 7.

Table 7 Conditions of the measurements of the eluting fractions of SPE with HPLC-ICPMS

Column	Zorbax 300-SCX (150 x 4.6 mm, 5 µm)
Mobile phases	10 mM pyridine buffer, pH 2.3 or 20 mM ammonium formate buffer, pH 2.5
Flow rate	1.5 mL/min
Injection volume	20 µL
Injection/draw speed	100 µL/min
Column compartment temperature	31°C

Retention of sodium

Sodium chloride (NaCl) is known to lead to signal-suppression in ESMS (see Figure 13). Unfortunately it occurs in high concentrations in urine. Hence we focused on sodium chloride to see how useful the clean-up with SPE has been. The measurements were performed with HPLC-ICPMS with the conditions given in Table 7.

The NaCl-experiments were only performed with the LiChrolut® SCX cartridge with different conditioning methods. Method one (formic acid pH 2.3), three (1 M HCl) and a fourth conditioning method with 100 mM HCl were used. Method two, using 6 M HCl was not used, because of the stability of the silica cartridge. Two blank SPE, one with method one and one with method three, were performed as well. For those preparations 1 mL ultrapure water was used in the loading step instead of 1 mL urine.

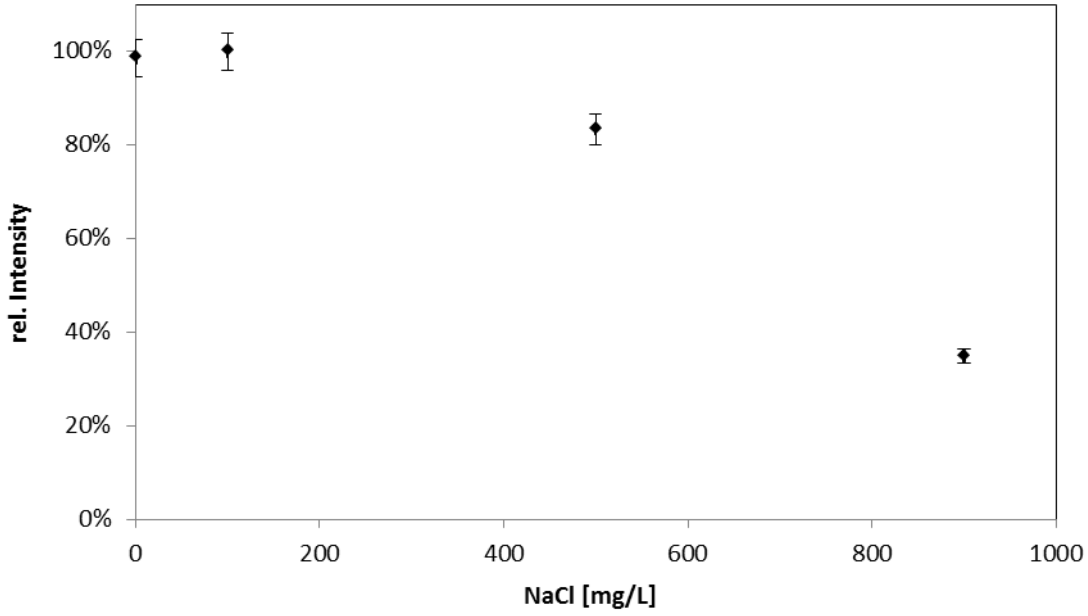


Figure 13 Signal intensity of AB depending on the NaCl concentration, measured with ESMS, Wolf Werner, University of Graz, Analytical Chemistry [89]

3.7.2 Optimization of the carbon enhancement effect in ICPMS

Arsenic is known to be hard to ionize under standard ICPMS conditions with a degree of ionization of 52 % [91]. Since we wanted to measure really low concentrations of arsenic we had to improve the method and try to enhance the degree of ionization by benefiting from the carbon enhancement effect.

To receive a maximal signal enhancement we investigated the best amount of carbon using 1 % CO_2 in Ar (v/v) as carbon donor and optional gas during the ICPMS measurement.

We prepared different solutions containing 1 µg/L AB and measured them with different amounts of optional gas (0-25 %). For every percent the optional gas flow was increased, the carrier gas flow was decreased by 0.01 L/min. The measured solutions were:

- Ultrapure water
- Ultrapure water with 1 % HNO_3
- 20 mM ammonium formate buffer, pH 2.5, adjusted with formic acid
- 10 mM pyridine buffer, pH 2.3, adjusted with formic acid

The samples were prepared by adding the appropriate amount of AB in one of the four solutions.

For monitoring the measurement, an internal standard, containing 200 µg/L Se, Te, In, Rb and Y, was added online via a t-piece in front of the nebulizer to all measured samples. The signal intensities of Se and Te were affected by the carbon content too. The signal intensity of In, Rb and Y should not show big differences during the whole measurement.

3.7.3 Selection of an adequate mobile phase for cation-exchange chromatography

For the separation of cationic arsenic species pyridine buffers are well known, but not really suitable for ESMS, because they lead to signal suppression. In this case, ammonium formate buffers seemed to be a good alternative.

To find the best mobile phase for our application, arsenic standards, in the range from 0.1 µg/L to 100 µg/L, containing DMA, MA, AB, As(III) and As(V) were measured with different mobile phases.

The first mobile phase was a 10 mM pyridine buffer at pH 2.3. An amount of 791 mg pyridine was diluted with 1 L ultrapure water and the pH was adjusted with formic acid to pH 2.3.

The second mobile phase was a 5 mM ammonium formate buffer at pH 2.5. We dissolved 315 mg ammonium formate in 1 L ultrapure water and adjusted the pH to pH 2.5 with formic acid.

The third mobile phase was a 5 mM ammonium formate buffer at pH 2.5 containing 10 % of methanol (v/v). We diluted 284 mg ammonium formate in 0.9 L ultrapure water, adjusted the pH at pH 2.5 with formic acid and mixed it with 100 mL methanol (MeOH).

All other conditions stayed the same and can be found in Table 7.

Retention of several cations

Na, K, Ca and Mg occur in high concentrations in urine. In ESMS Na and K are known to lead to signal suppression, therefore it was important to separate them from AB. To proof this, urine sample 32307, containing AB, was measured with HPLC-ICPMS focusing on these 4 elements. Because former experiments have shown, that a 20 mM ammonium formate buffer seemed to be the mobile phase of choice, this experiment was only performed with this mobile phase. Further conditions can be found in Table 7.

3.7.4 Optimization of the mobile phase for HPLC-ESMS

We wanted to measure AB directly in urine samples, as sensitive as possible. For speciation analysis with HPLC-ICPMS we used a 20 mM ammonium formate buffer at pH 2.5. We dissolved 1.26 g (20 mM) ammonium formate in 1 L ultrapure water and adjusted the pH with formic acid to pH 2.5. This worked well with ICPMS, when working with ESMS an amount of organic solvent can lead to better ionization of the compounds. Furthermore the content of organic solvent affects the chromatographic separation. When measuring urine samples directly with ESMS it is good to shift the analyte of interest between the matrix compounds to minimize signal suppression.

To optimize the ionization and retention of AB we used urine samples spiked with 80 µg/L DMA, 10 µg/L MA, 100 µg/L AB, 5 µg/L As(III) and 5 µg/L As(V); to simulate a urine after fish consumption. We measured a spiked urine sample with different amounts of MeOH, in the mobile phase, to observe which MeOH content leads to the best signal of AB.

The settings for the HPLC can be found in Table 7 with the exception of the mobile phase. In this experiment we used a 20 mM ammonium formate buffer (pH 2.5) with different amounts of MeOH (0-80 %). The settings for the ESMS can be found in Table 8.

Because a flow rate of 1.5 mL/min with an aqueous buffer is too high for ESMS we split the flow after the HPLC 1:1 (seen Figure 14). The capillary to the ESMS and to the waste had the same length and diameter to obtain a 1:1 splitting.

Table 8 Instrument settings for LC-ESMS for the optimization of the methanol content

Polarity	positive
Drying gas flow	13 L/min
Nebulizer pressure	35-60 psig
Drying gas temperature	350°C
Capillary voltage	Positive: 4000 V Negative: 4000 V
Fragmentor voltage	100 V
Scan (MSD1) [m/z]	70-300
SIM (MSD2) [m/z]	75, 139, 141, 179

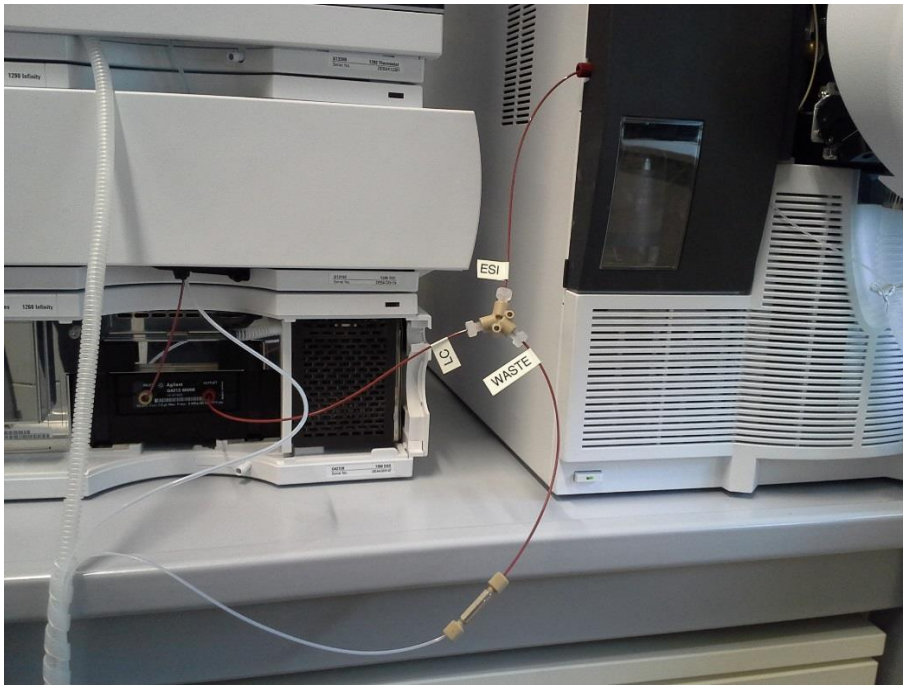


Figure 14 Post-column splitting in HPLC-ESMS

3.8 USG - a good way of normalisation?

To get a better impression of the USG a simple dilution experiment was performed. Two urine samples, with a quite high USG (sample 12204, USG = 1.021 and sample 42204, USG = 1.024) were diluted with ultrapure water and the USG was measured with the total solids refractometer to proof, how good the USG correlates to the dilution of the urine. The measured samples ranged from 100 % urine (not diluted) to 5 % urine (diluted 1 + 19). Each dilution step was prepared 5 times, to exclude dilution and measurement errors.

3.9 Determination of Total Element Concentrations

We measured the total arsenic concentrations as well as the total concentrations of other elements of the urine samples with an ICPMS 7700x from Agilent. As our focus was on arsenic, we measured all samples with 16 % optional gas (1 % CO_2 in Ar (v/v)), to benefit from the carbon enhancement effect. Some elements were measured in the nogas-mode and others in the collision mode, in which 4 mL/min He were used as collision gas (see Table 9).

For quantification we used external calibrations. Because the ranges of the different elements were quite different we used single element standards. Some elements were present in very high concentrations, which made it necessary to prepare a second set of calibration standards, containing these elements (Ca, Cu, Fe, K, Mg, Na, P and S) (see Table 9). All calibration standards were prepared by diluting stock solutions with ultrapure water and 10 % (v/v) nitric acid.

For quality control we used certified reference materials: SRM® 1640a and Seronorm™. Both certified reference materials were diluted 1 + 9 with ultrapure water and 10 % (v/v) nitric acid.

Furthermore we used drift standards for quality control. A calibration standard, located in the middle of the calibration range, was re-measured approximately all 10 samples to observe the stability of the run.

Another method to observe the stability of the run was the addition of an internal standard. This standard contained 200 µg/L Be, Ge, In and Lu and was added online via a t-piece before the nebulizer to all measured samples (including calibration standards and certified reference materials). A change in the signal of the internal standard indicates quite often a change in the sample matrix. The signals of all elements were corrected by the internal standard. The corresponding elements of the internal standard used for the signal correction of the measured elements can be found in Table 9.

Table 9 Settings for ICPMS measurement of the total amount of different elements; m/z of internal standards: Be = 9, Ge = 74, In = 115, Lu = 175

Element	m/z	Octopole mode	Internal standard	Calibration range
As	75	He	Ge	0.1 - 500 µg/L
B	11	nogas	Be	0.1 - 10 µg/L
Ba	137	nogas	In	0.01 - 1 µg/L
Bi	209	nogas	Lu	0.01 - 1 µg/L
Ca	43	nogas	Be	0.1 - 10 mg/L
Cd	111	nogas	In	0.01 - 1 µg/L
Co	59	He	Be	0.01 - 1 µg/L
Cr	52	He	Be	0.01 - 1 µg/L
Cu	65	He	Ge	0.1 - 10 mg/L
Fe	56	He	Be	0.1 - 5 mg/L
Hg	201	nogas	Lu	0.01 - 1 µg/L
K	39	He	Be	1 - 100 mg/L
Li	7	nogas	Be	0.01 - 1 µg/L
Mg	24	nogas	Be	0.1 - 10 mg/L
Mn	55	He	Be	0.01 - 1 mg/L
Mo	98	nogas	In	0.01 - 1 µg/L
Na	23	He	Be	1 - 100 mg/L
Ni	60	He	Ge	0.01 - 1 µg/L
P	31	He	Be	1 - 100 mg/L
Pb	208	nogas	Lu	0.01 - 1 µg/L
Rb	85	He	Ge	0.01 - 1 mg/L
S	34	He	Be	1 - 100 mg/L
Sb	121	nogas	In	0.01 - 1 µg/L
Se	82	nogas	Ge	0.1 - 10 µg/L
Sr	88	He	Ge	0.01 - 1 µg/L
Te	125	nogas	In	0.01 - 1 µg/L
U	238	nogas	Lu	0.01 - 1 µg/L
V	51	He	Be	0.01 - 1 µg/L
Zn	66	He	Ge	0.1 - 10 mg/L

3.10 Arsenic Speciation Analysis

We used two systems for arsenic speciation. For cation-exchange chromatography we used a HPLC 1260 Infinity coupled to an ICPMS 7500ce from Agilent. For anion-exchange we used a HPLC 1200 coupled to an ICPMS 7700x from Agilent. The change of the measuring system was only because of its availability. The ICPMS was used in collision mode, using 4 mL/min He. Furthermore 16 % optional gas (1 % CO_2 in Ar (v/v)) were used to enhance the arsenic signal. The autosampler of the HPLC was always kept at 4°C. The stock solutions for the calibration standards were stored in the fridge at 4°C. The calibration standards were prepared on the day of the corresponding measurement. The mobile phases were prepared in 2.5 L plastic bottles to avoid contaminations from glass bottles. The calibration standards and the samples were prepared in 15 mL PP tubes and measured in 0.7 mL PP vials.

3.10.1 Cation-exchange chromatography

We used our optimized cation-exchange chromatography method to determine the different arsenic species, especially AB and other cationic species that may occur in the urine samples. We prepared standard solutions by mixing As(III), As(V), DMA, MA and AB and diluting them with ultrapure water. The calibration ranged from 0.1 to 250 µg As/L for each arsenical.

We prepared the mobile phase by solving 1.26 g (20 mM) ammonium formate in 1 L ultrapure water and adjusted the pH with formic acid to pH 2.5.

The typical conditions for cation-exchange chromatography can be found in Table 7 with 20 mM ammonium formate buffer, pH 2.5, as mobile phase and a typical pressure of 100-110 bar. A typical chromatogram of a calibration standard, containing As(III), As(V), DMA, MA and AB is shown in Figure 15.

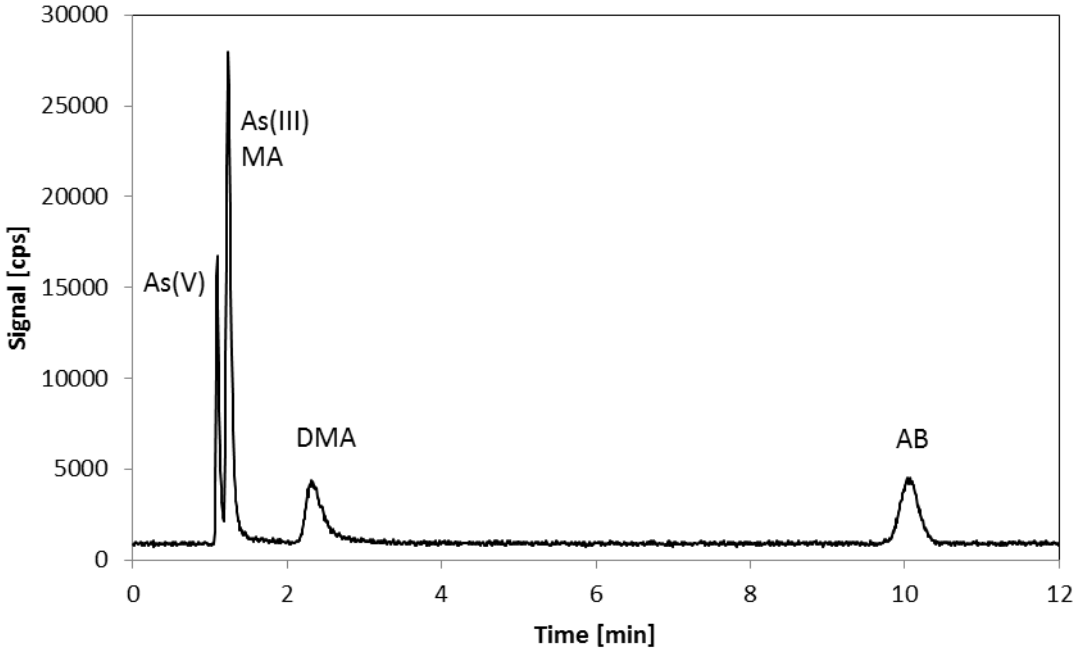


Figure 15 Cation-exchange chromatogram of a standard solution containing 10 µg/L As of As(III), As(V), DMA, MA and AB, injection volume = 20 µL, measured with 20 mM ammonium formate buffer, pH 2.5, as mobile phase and a Zorbax 300-SCX (150 x 4.6 mm, 5 µm) column as stationary phase, with HPLC-ICPMS (16 % optional gas (1 %CO_2 in Ar (v/v))), in the collision mode (4 mL/min He)

3.10.2 Anion-exchange chromatography

To prove the results of the cation-exchange chromatography and to make sure not to miss any unexpected arsenical, we measured all urine samples with anion-exchange chromatography too. We used the method described by Scheer et al. [92].

We prepared standard solutions by mixing As(III), As(V), DMA, MA and AB diluting them with ultrapure water and 10 % v/v of a 30 % H_2O_2 solution. We let the standards oxidize about 2-3 hours at 50°C. The calibration ranged from 0.1 to 250 µg As/L for each arsenical.

We prepared the mobile phase by diluting 2.31 g (20 mM) orthophosphoric acid in 1 L ultrapure water and adjusted the pH with NH_4OH to pH 6.0.

For quality control we used certified reference materials: SRM® 1640a and Seronorm™. Both certified reference materials were diluted 1 + 9 with ultrapure water and oxidized with a 10 % v/v of a 30 % H_2O_2 solution, for ~2-3 hours at ~50°C, before measuring.

A typical chromatogram of a calibration standard is shown in Figure 16 and further measurement conditions can be found in Table 10.

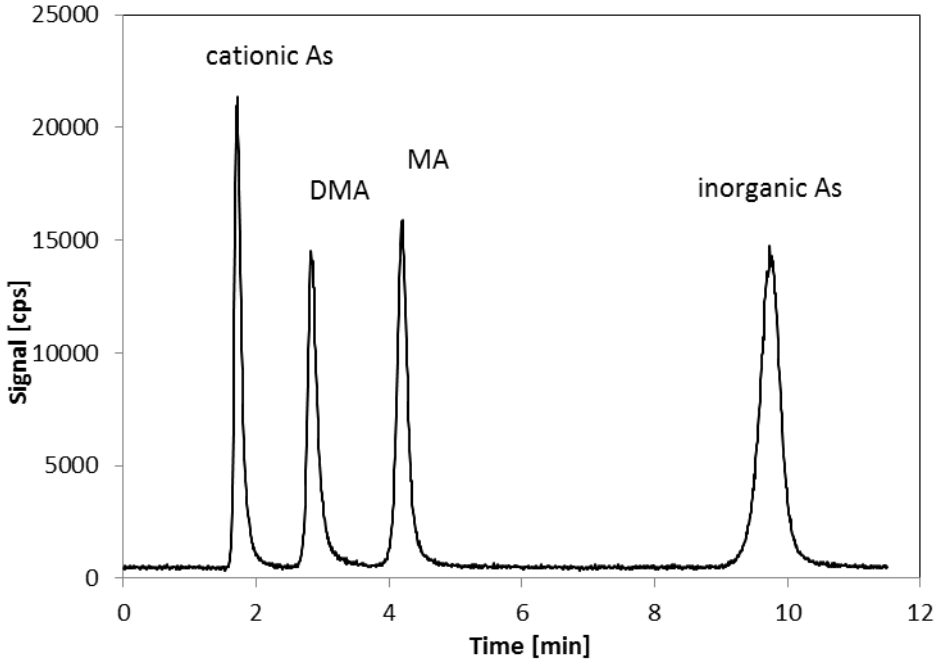


Figure 16 Anion-exchange chromatogram of a standard solution containing 10 µg As/L of AB, DMA, MA, As(III) and As(V), oxidized, with a 20 mM ammonium phosphate buffer, pH 6.0, as mobile phase and a PRP-X100 (150 x 4.6 mm, 5 µm) column with a PRP-X100 (8 x 3 mm, 10 µm) guard column as stationary phase, injection volume = 20 µL, measured with HPLC-ICPMS (16 % optional gas (1 %CO_2 in Ar (v/v))), in the collision mode (4 mL/min He)

Table 10 Condition of the anion-exchange chromatography for arsenic species analysis in urine samples

Column	PRP-X100 (150 x 4.6 mm, 5 µm)
Guard column	PRP-X100 (8 x 3 mm, 10 µm)
Mobile phase	20 mM ammonium phosphate buffer, pH 6.0
Flow rate	1 mL/min
Injection volume	20 µL
Injection/draw speed	100 µL/min
Column compartment temperature	40°C
Typical pressure	85-100 bar

3.11 Molecular Mass Analysis

For molecular mass analysis we used a 6120 quadrupole LC/MS. The autosampler of the HPLC was always set to 4°C. The stock solutions for the calibration standards were stored in the fridge at 4°C. The calibration standards were prepared on the day of the corresponding measurement. The buffer used for the mobile phases was prepared in 2.5 L plastic bottles. The calibration standards and the samples were prepared in 15 mL PP tubes and measured in 0.7 mL PP vials.

The settings of the HPLC were the same for all LC-ESMS measurements. The conditions can be found in Table 11.

Table 11 HPLC instrument settings for LC-ESMS

Column	Zorbax 300-SCX (150 x 4.6 mm, 5 µm)
Mobile phase	20 mM ammonium formate buffer, pH 2.5 + 65 % (v/v) MeOH
Flow rate	1.5 mL/min (split 1:1 post column)
Injection volume	20 µL
Injection/draw speed	100 µL/min
Column compartment temperature	31°C
Typical pressure	165-200 bar

3.11.1 Signal suppression caused by the urine matrix

The urine matrix leads to signal suppression in ESMS. The influence of the matrix in our chromatographic method has to be investigated.

Ultrapure water and a urine sample with a high specific gravity (sample 12204, USG = 1.021) were spiked with different amounts of AB. The spiking was done by the autosampler of the HPLC. 18 µL of the sample were mixed with 2 µL of an adequate AB standard. The different AB standards were prepared by diluting the stock-solution with a proper amount of ultrapure water.

The ESMS instrument settings can be found in Table 12.

Table 12 Instrument settings for ESMS for the determination of signal suppression caused by the urine matrix

Polarity	positive
Drying gas flow	13 L/min
Nebulizer pressure	35-60 psig
Drying gas temperature	350°C
Capillary voltage	Positive: 4000 V Negative: 4000 V
Fragmentor voltage	100 V
Scan (MSD1) [m/z]	150-200
SIM (MSD2) [m/z]	75, 179, 182, 185, 188

Another investigation was the influence of the USG on the signal suppression in ESMS. A urine sample with a high specific gravity (sample 12204, USG = 1.021), the same sample as before, was diluted stepwise with ultrapure water and spiked with 10 µg/L d^9AB, to investigate the influence of the dilution of the urine matrix. The dilution steps were 99 + 1, 49 + 1, 19 + 1, 4 + 1 and 1 + 1 (urine + ultrapure water). One sample containing only ultrapure water and 10 µg/L d^9AB was measured as well. To compare the results with other urine samples the USG of each sample and the urine without dilution were measured.

The ESMS instrument settings can be found in Table 12. Only the settings for the MSD 1 (m/z = 50-250) and MSD2 (m/z = 61, 156 and 188) were different.

3.11.2 Urine matrix analysis

The urine matrix contains many different molecules and ions. As ESI is easily suppressed by many of these compounds, we focused on the urine matrix. The influence of different metal ions (Na^+, K^+, Mg^{2+}…) as well as the major organic compounds (urea, uric acid...) were investigated.

For the preparation of the solutions containing a matrix compound the chosen concentration was set to the upper boundary of the range of the typical urinary amount. Each compound was dissolved in ultrapure water and the autosampler of the HPLC spiked each sample, as well as ultrapure water, with 10 µg/L AB.

The different compounds and their used concentrations can be found in Table 13. The instrument settings can be found in Table 12. In the SIM mode (MSD1) always m/z = 179 (AB) and sometimes a m/z corresponding to the matrix compound were detected. The MSD2 was set to m/z = 50-250.

Table 13 Compounds used in urine matrix experiments

Matrix compound	Concentration [g/L]	Source	m/z in ESMS
Na^+	3.9	NaCl	
K^+	0.55	KCl	
Ca^{2+}	0.25	$CaCl_2 \cdot 2H_2O$	
Mg^{2+}	0.13	$MgCl_2 \cdot 6H_2O$	
NH_4^+	0.73	NH_4Cl	
Cl^-	5.9	NH_4Cl	
PO_4^{3-}	2.7	$(NH_4)_2HPO_4$	
SO_4^{2-}	2.0	$(NH_4)_2SO_4$	
Urea	13	Urea	61
Uric acid	0.33*	Uric acid	169
Glycine	0.21	Glycine	76
Lysine	0.10	Lysine	147
Glutamine	0.11	Glutamine	147
Histidine	0.30	Histidine	156
Sodium oxalate	0.066	Sodium oxalate	

***** not totally dissolved

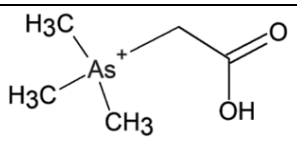
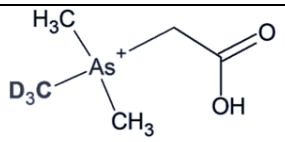
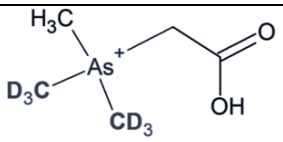
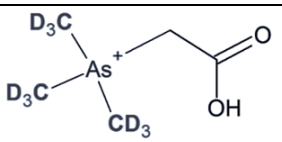
3.11.3 Urine study

Some organic compounds containing nitrogen exist as arsenic analogues like arsenosugars, arsenic containing hydrocarbons, arsenolipids and AB as well. This is because of the similarity of the two elements. Therefore, a urine study investigating whether AB is metabolized in the human body similar to GB, was performed.

Five volunteers, 3 women and 2 men in the age of 17 to 48 years ingested 2 mg of arsenic in form of d^9AB. One week before and during the study the volunteers did not consume any seafood avoiding high background levels of arsenic. Starting one day before the ingestion till the end of the study, the volunteers recorded their ingested food and drinks and collected their urine. For every collection the time of the urination and the amount of urine were recorded and an aliquot of 40 mL was stored in the fridge for measurements. The details of each urine sample can be found in Table 29 in the appendix.

The study was performed to investigate, if AB can act as a methyl-donor in the one carbon cycle, like GB. To record any changes of the deuterated arsenic species, molecular mass spectrometry (ESMS) was used to measure m/z = 179 (non-deuterated AB), 182, 185 (partly-deuterated) and 188 (d^9AB) in the urine samples (see Table 14).

Table 14 AB with different deuterium content; on the left side the common non-deuterated form (AB) and on the very right the ingested deuterated form (d^9AB)

m/z = 179	m/z = 182	m/z = 185	m/z = 188
			

After the ingestion of d^9AB (m/z = 188) we expected high amounts of this compound in the urine samples. Non-deuterated AB (m/z = 179) might occur as well and could have been ingested with the diet. The occurrence of partly deuterated AB (m/z = 182 and 185) would indicate a reaction of d^9AB in the human body and possibly imply that AB can act as a methyl-donor in the one carbon cycle.

Because ESMS is known to lead to signal suppression when more than one compound enters the ES-source at the same time, a pre-experiment was performed. The four different deuterated ABs should elute at the same time and each of them could suppress the others. To investigate the influence of those suppressions, three calibrations ranging from 1-100 µg/L AB were prepared. The first calibration contained only AB, the second only d^9AB and the third both, AB and d^9AB. The calibration standards were prepared by diluting the

proper amount of AB or d^9AB with ultrapure water. The integrated areas of the calibrations containing only one form of AB (AB or d^9AB) were compared with the areas in the calibration containing both forms of AB to prove if they suppress each other.

The instrument settings for the ESMS can be found in Table 12. The MSD1 was set at m/z = 50-210 and the MSD2 at the m/z from the 4 different ABs (m/z = 179, 182, 185 and 188).

To investigate if AB can act as a methyl-donor in the one carbon cycle an external calibration, containing AB and d^9AB in a range from 0.1-500 µg/L was prepared by diluting the proper amount of the stock-solutions with ultrapure water. Then all urine samples were measured with HPLC-ESMS focusing on m/z = 179, 182, 185 and 188. The Instrument settings were the same as mentioned above.

To get a better impression of these results, the urine samples with the highest concentration of d^9AB from each person, were spiked with AB to determine the amount that is still observable. The spiking was done by the autosampler by mixing 19 µL of the urine sample with 1 µL of a proper standard solution. We started with a spike resulting in 5 µg/L AB to be sure to observe a peak. Further spikes resulted in 1 µg/L, 0.5 µg/L, 0.3 µg/L and finally 0.1 µg/L AB. Every concentration was spiked 3 times.

The standard solutions were prepared by diluting a proper amount of the stock-solution with ultrapure water. The instrument settings were the same as mentioned above.

4 Results and Discussion

4.1 Method Development

4.1.1 Selection of an adequate cartridge material and conditioning method for SPE

Bond Elut Plexa PCX SPE-cartridge

With the Bond Elut Plexa PCX no big differences between method one (formic acid) and two (6 M HCl), method three was not tested, could be observed. In both cases AB retained on the cartridge and eluted quite fast with 100 mM ammonium formate (see Figure 17 and Figure 18). In the first five fractions nearly all AB eluted from the cartridge. The ultrapure water from the washing steps was collected in two fractions. The first 4 mL were collected together in one fraction and the fifth one in another one. Figure 17 and Figure 18 show that all other arsenic species started eluting during the washing step. Only AB retained on the cartridge and eluted nearly completely in the first eluting fraction.

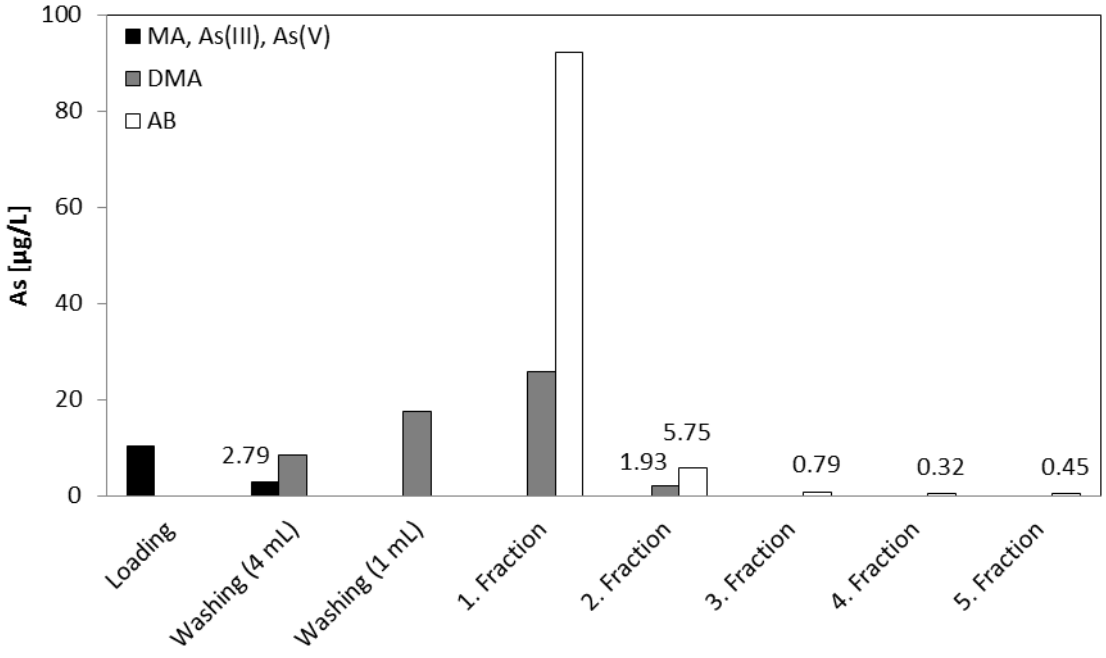


Figure 17 Eluting fractions from SPE with the Bond Elut Plexa PCX cartridge conditioned with formic acid (pH 2.3), 1 mL each fraction, measured with HPLC-ICPMS

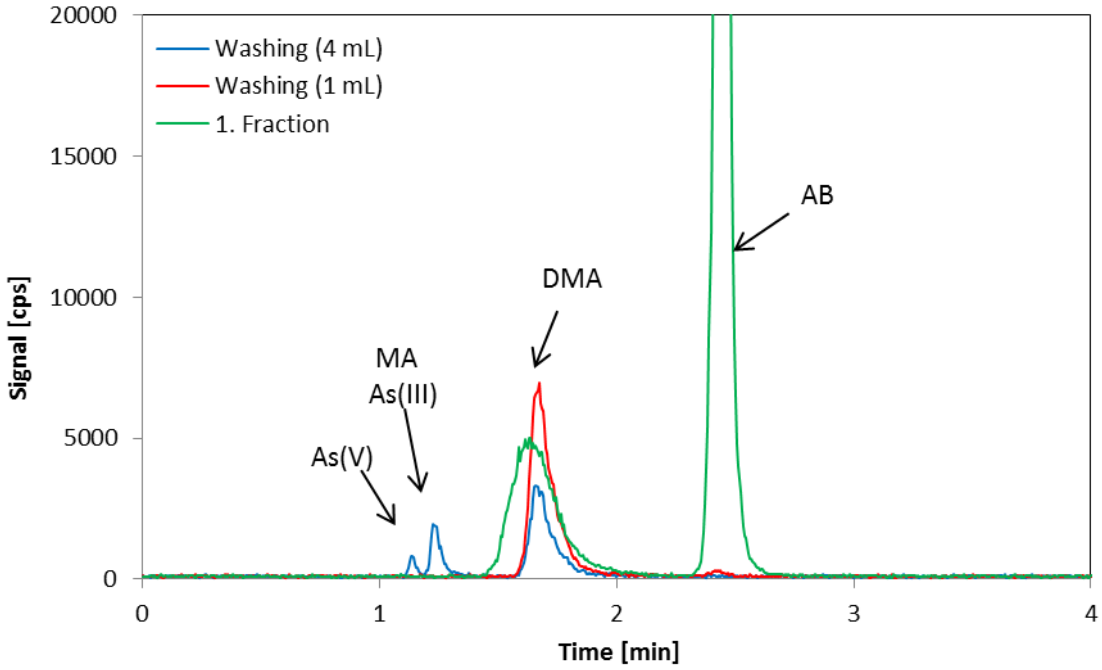


Figure 18 Chromatogram of different eluting fractions of SPE with the Bond Elut Plexa PCX, SPE conditioned with formic acid (pH 2.3), measured with HPLC-ICPMS

With both conditioning methods the recoveries of all five arsenic species were good. Because MA, As(III) and As(V) were not baseline separated they were integrated together to avoid possible errors. The concentrations after loading, washing and seven eluting fractions (fraction six and seven eluted with 1 M ammonium formate) are summarized in Table 15.

Table 15 Results of the SPE with the Bond Elut Plexa PCX with different conditioning methods after 7 eluting fractions

	Formic acid, pH 2.3			**6 M HCl**		
	MA, As(III), As(V)	**DMA**	**AB**	**MA, As(III), As(V)**	**DMA**	**AB**
Sum [µg/L]:	21.4	79.0	100	19.6	77.2	101
Recovery [%]	>99	95	97	91	93	98

By measuring the spiked urine without clean-up 21.4 µg/L of MA, As(III) and As(V), 83.4 µg/L DMA and 103 µg/L AB were found. This is more than the added amount of each arsenical and occurs from an arsenic background in the used urine.

LiChrolut® SCX SPE-cartridge

With the LiChrolut® SCX cartridge larger differences between the three conditioning methods could be observed. Similar to the Bond Elut Plexa PCX cartridge DMA, MA, As(III) and As(V) started eluting in the washing step and only AB retained on the cartridge (see Figure 19). Surprisingly no AB eluted in the first eluting fraction, neither with method one, two or three.

Furthermore, with the LiChrolut® the highest amount of AB was not observed in the first fraction containing AB (see Figure 20).

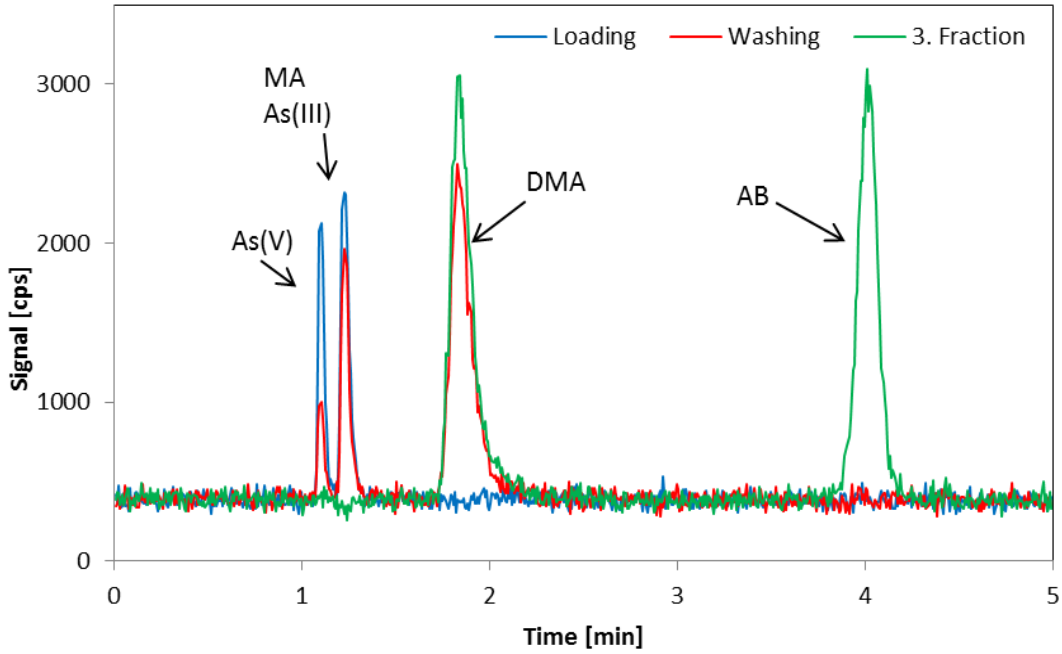


Figure 19 Chromatogram of different fractions of SPE with the LiChrolut® SCX, conditioned with formic acid (pH 2.3), measured with HPLC-ICPMS

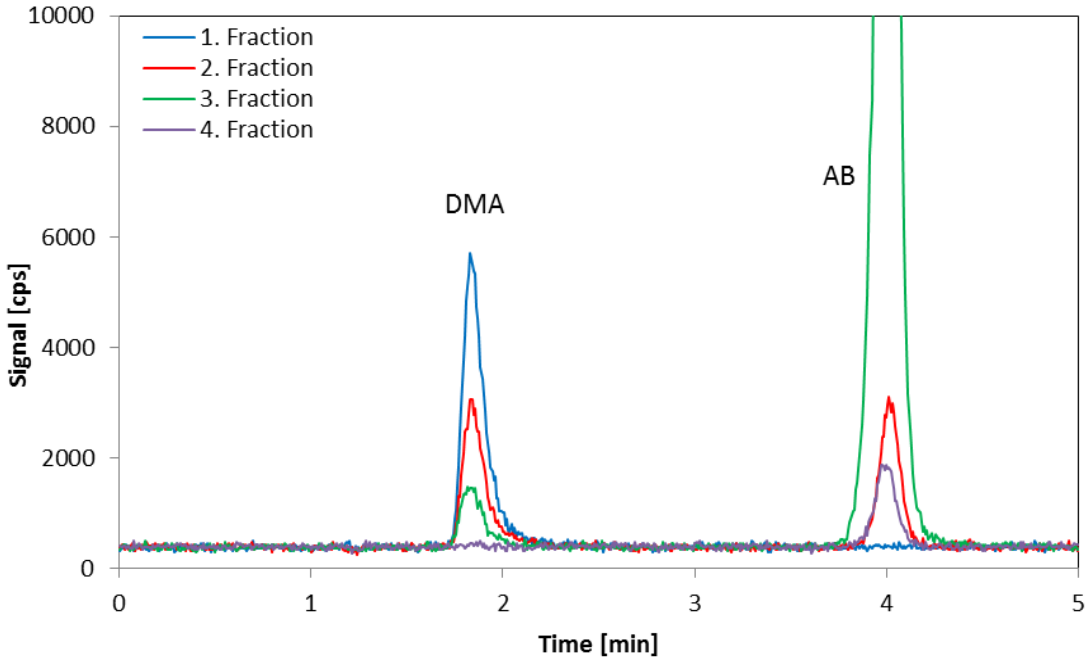


Figure 20 First four eluting fractions of the SPE with the LiChrolut® SCX, conditioned with formic acid (pH 2.3), measured with HPLC-ICPMS

With the two HCl-methods AB was present in more fractions than with the formic acid method. With all three methods good recoveries for all arsenic species could be received. 0.33 µg/L MA, As(III) and As(V), 0.50 µg/L DMA and 0.24 µg/L AB could be observed in the used non-spiked urine as background values. The results of the different fractions and the different methods are summarized in Table 16 to Table 18 and Figure 21.

Table 16 Results of MA, As(III) and As(V) after SPE with the LiChrolut® SCX

Conditioning method	**Formic acid pH=2.3** MA, As(III), As(V) [µg/L]	**6 M HCl** MA, As(III), As(V) [µg/L]	**1 M HCl** MA, As(III), As(V) [µg/L]
Loading	5.12	4.20	3.85
Washing (5 mL)	2.98	3.02	3.18
Sum	**20.0**	**19.3**	**19.7**
Recovery [%]	**98**	**95**	**97**

Table 17 Results of DMA after SPE with the LiChrolut® SCX, 1 mL each fraction

Conditioning method	**Formic acid pH=2.3** DMA [µg/L]	**6 M HCl** DMA [µg/L]	**1 M HCl** DMA [µg/L]
Loading	-	-	-
Washing (5 mL)	7.99	0.72	-
1. Fraction	21.0	2.71	1.23
2. Fraction	10.7	3.23	2.85
3. Fraction	4.96	10.7	5.06
4. Fraction	-	19.7	14.1
5. Fraction	-	36.7	23.9
6. Fraction	-	2.71	25.9
7. Fraction	-	-	4.50
Sum	**76.6**	**79.3**	**77.5**
Recovery [%]	**95**	**98**	**96**

Table 18 Results of AB after SPE with the LiChrolut® SCX, 1 mL each fraction

Conditioning method	**Formic acid pH=2.3** AB [µg/L]	**6 M HCl** AB [µg/L]	**1 M HCl** AB [µg/L]
1. Fraction	-	-	-
2. Fraction	8.33	1.77	-
3. Fraction	83.5	15.9	3.71
4. Fraction	5.48	30.5	22.5
5. Fraction	-	46.3	30.9
6. Fraction	-	4.02	33.0
7. Fraction	-	-	5.96
Sum	**97.3**	**98.4**	**96.0**
Recovery [%]	**97**	**98**	**96**

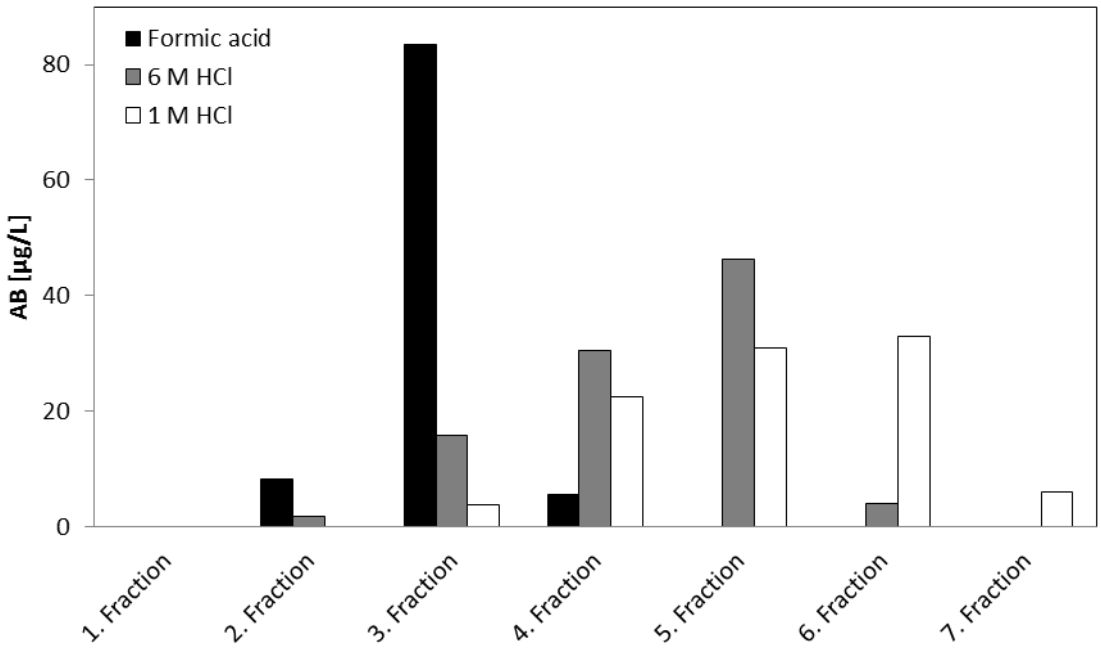


Figure 21 Distribution of AB in the eluting fractions of SPE with different conditioning methods with the LiChrolut® SCX cartridge, 1 mL each fraction, measured with HPLC-ICPMS

Retention of sodium

The rather unexpected results of the sodium concentrations are listed in Table 19. In the blank SPE 1 mL of ultrapure water was used instead of 1 mL urine in the loading step.

Table 19 Distribution of sodium in the elution fractions of SPE with the LiChrolut® SCX and different conditioning methods, in the blanks 1 mL of ultrapure water was used instead of 1 mL urine in the loading step, each fraction was 1 mL

Method	**Blank formic acid** Na [mg/L]	**Blank 1 M HCl** Na [mg/L]	**Formic acid** Na [mg/L]	**1 M HCl** Na [mg/L]	**100 mM HCl** Na [mg/L]
Loading	194	1.86	409	-	124
Washing (5 mL)	53.2	-	400	-	83.1
1. Fraction	68.3	-	62.7	-	3.07
2. Fraction	257	-	454	-	14.8
3. Fraction	1130	-	1430	34.6	56.2
4. Fraction	2030	-	1630	140	151
5. Fraction	1340	-	956	129	287
6. Fraction	318	-	325	90.2	101
7. Fraction	7.86	-	66.4	60.3	0.49
Sum	**5390**	**1.86**	**5730**	**454**	**820**
% of Urine	**810**	**0.28**	**870**	**69**	**120**

The results from the SPE with the formic acid method, listed in Table 19, seemed a bit implausible. Even in the blank cartridge more than 800 % of sodium, compared to the sodium content of the urine itself, could be observed. This means 8 times more sodium was detected after the clean-up than in the urine itself. Such high sodium concentration in the formic acid samples can only occur due to a contamination of the cartridge. This assumption correlates with the measurement. When the cartridge was conditioned very acidic, no contamination could be observed, because the whole sodium already eluted in the conditioning step. The less acidic the conditioning step was, the more sodium was present in the further steps. This would explain why high concentrations of sodium could be observed when the conditioning step was performed with 100 mM HCl as well. To support this hypothesis a further experiment was performed in which only one cartridge was used for 4 clean-ups. In the first two extractions ultrapure water and in the third and fourth urine was used in the loading step. This experiment was performed only with formic acid as conditioning agent and the conditioning steps were collected as well, always 5 mL together. The results are shown in Figure 22.

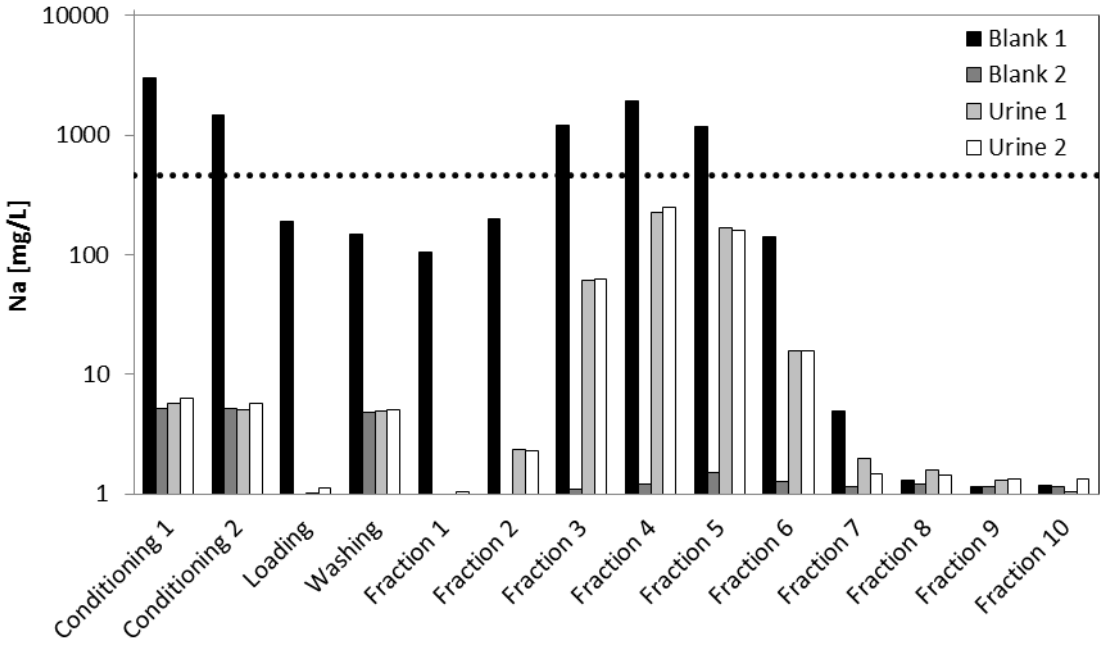


Figure 22 Sodium concentrations in SPE fractions with the LiChrolut® SCX conditioned with formic acid (pH 2.3), 1 mL each fraction, measured with ICPMS; the dotted line is the sodium concentration of the urine sample

In the first blank clean-up high concentrations of sodium could be observed (see Figure 22). In the second blank clean-up nearly no sodium could be observed any more. Two blank clean-ups should be enough to clean the cartridge. Figure 23 shows that sodium eluted nearly in the same fractions as AB in the two urine clean-ups.

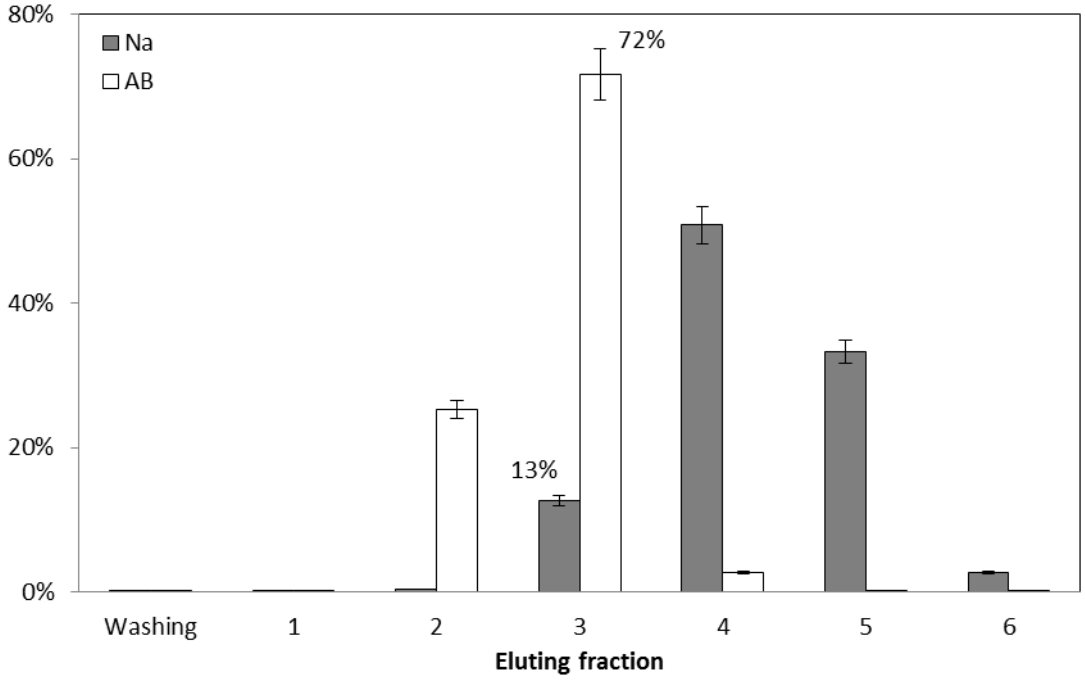


Figure 23 Comparison of the retention behaviour of Na and AB on the LiChrolut® SCX cartridge conditioned with formic acid (pH 2.3), 4 mL washing and 1 mL each fraction, measured with HPLC-ICPMS, 100 % is the total amount of each compound

As seen in Figure 23 there is a slight difference of the retention behaviour of Na and AB. In the main fraction of AB (fraction 3) only 13 % of Na elute. For this reason a clean-up with the LiChrolut® SCX cartridge conditioned with formic acid (pH 2.3) seemed to be the method of choice.

4.1.2 Optimization of the carbon enhancement effect in ICPMS

First the signals of AB in two mobile phases used in cation-exchange chromatography were compared. The highest signals were obtained with 15 % optional gas (1 % CO_2 in Ar (v/v)) with both mobile phases (see Figure 24). At lower concentrations of CO_2 the signals of AB in the pyridine buffer were higher than in the ammonium formate buffer. At higher CO_2 concentrations the signal intensities were nearly the same. Consequently the signal enhancement in the ammonium formate matrix (factor 2.3) was higher than in the pyridine matrix (factor 2). When comparing the two buffer systems their carbon content is an important parameter. The 10 mM pyridine buffer contains ~50 mmol C/L and the 20 mM

ammonium formate buffer contains ~20 mmol C/L. The higher carbon content of the pyridine buffer led to a higher signal of AB without the addition of further carbon with the optional gas. High amounts of additional carbon led to a saturation of the enhancement and minimized the differences between the buffers. Figure 24 demonstates quite well, that an addition of high amounts of carbon via the optional gas can minimize the influence of carbon containing matrices for the measurement of As with ICPMS.

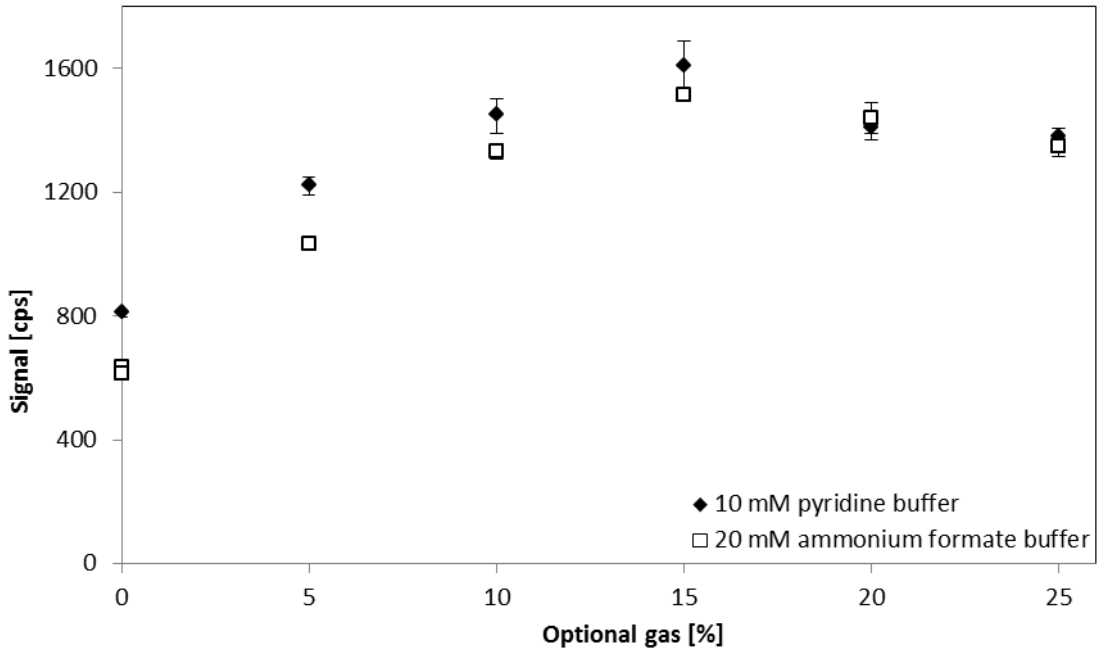


Figure 24 Optimization of the carbon enhancement effect by measuring 1 µg/L AB with ICPMS in two mobile phases (used in speciation), with different amounts of optional gas (1 % CO_2 in Ar (v/v))

Comparing the signal enhancement of AB in ammonium formate buffer with AB in ultrapure water; a higher enhancement could be observed in water (see Figure 25). As expected, the signal of AB without optional gas was higher in the ammonium formate buffer, because the buffer contained already a certain amount of carbon. Furthermore the addition of small amounts of carbon led to a stronger effect in water and therefore a higher enhancement in water than in the buffer solution.

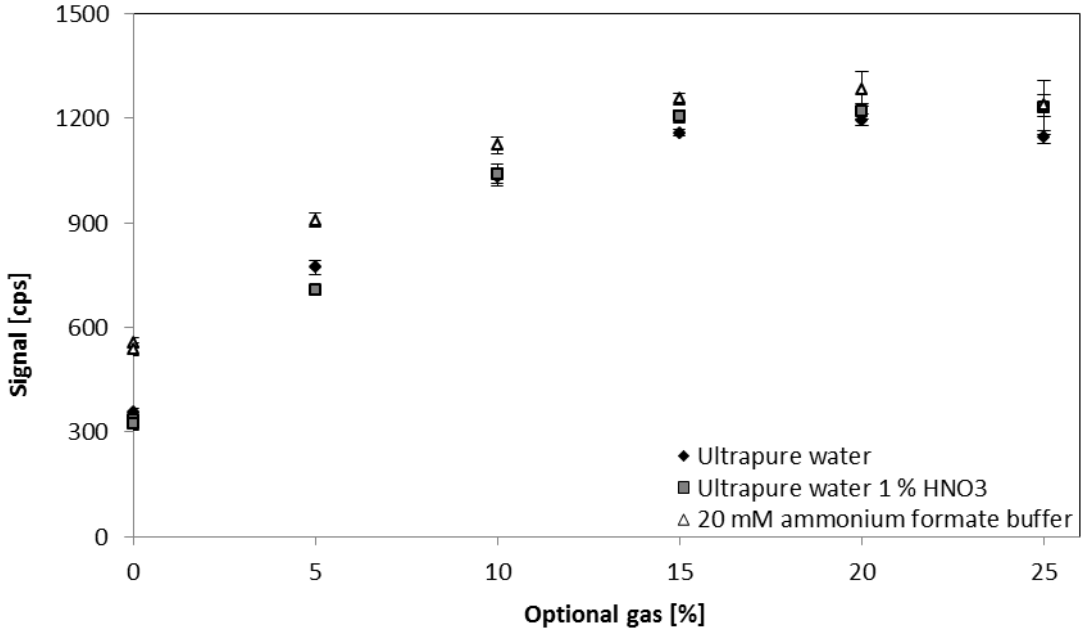


Figure 25 Comparison of the carbon enhancement effect in ammonium formate buffer and ultrapure water by measuring 1 µg/L AB in ultrapure water or ammonium formate buffer with ICPMS with different amounts of optional gas (1 % CO_2 in Ar (v/v))

In Figure 25 the highest signal was not at 15 % optional gas as in Figure 24. In this second experiment the highest signal intensity could be observed somewhere between 15 and 20 %. Because of the small differences, an optional gas flow of 16 % was used in further measurements. Comparing ultrapure water and the ammonium formate buffer, the enhancement factor in ultrapure water, a non-carbon-matrix, was higher. Because of the carbon content of the ammonium formate (20 mmol) and the pyridine buffer (50 mmol) the addition of further carbon did not lead to such high enhancement. In the ultrapure water matrices a signal enhancement of a factor of 3.5 could be observed in contrast to the ammonium formate buffer with a signal enhancement factor of 2.3.

The signal intensities of the internal standard behaved as expected. Se and Te, two elements which are also affected by the carbon enhancement effect, showed the same trend as As. In Figure 26 the signals from Se and Te in a 20 mM ammonium formate buffer are shown. The highest signal intensities for Se and Te were observed at 15 % optional gas. This maximum corresponds to the highest intensity evaluated for As (Figure 24).

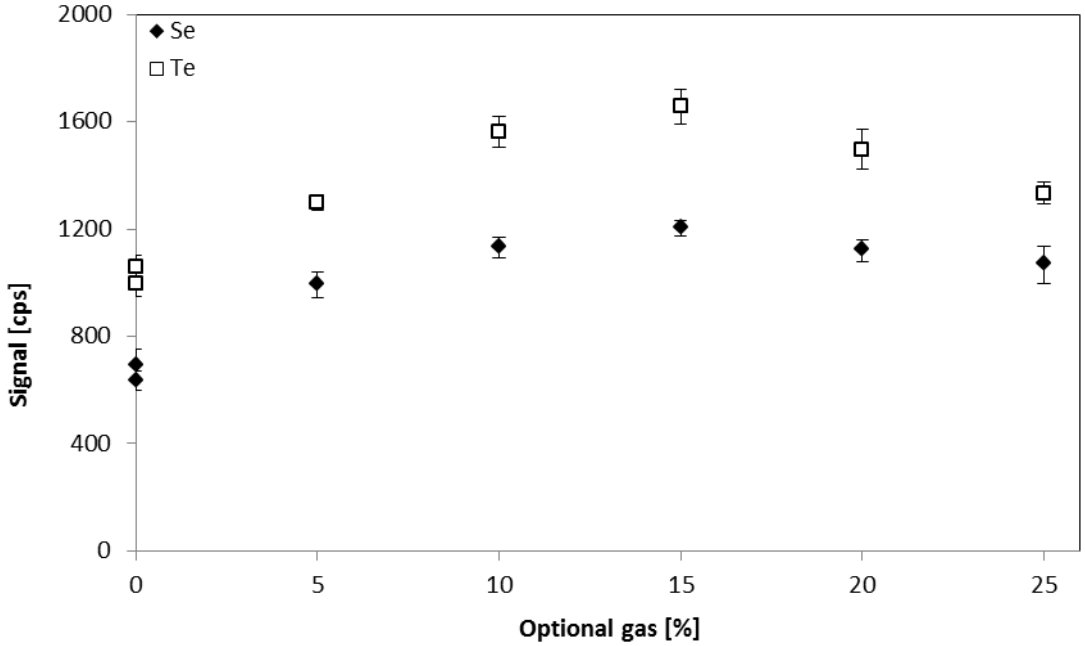


Figure 26 Carbon enhancement effect of Se and Te used as internal standard in a 20 mM ammonium formate buffer matrix, measured with ICPMS with different amounts of optional gas (1 % CO_2 in Ar (v/v))

Rb, Y and In showed a dependency of the carbon content too (Figure 27). With higher carbon content the signal intensities decreased for about 30 %. A technical mistake could be eliminated because the signal intensities at 0 % optional gas were the same at the beginning and at the ending of the measurement. One explanation for this observation is the variation of the plasma temperature. With higher amounts of optional gas the plasma temperature decreases, the atoms are not totally ionized anymore and the signal decreases.

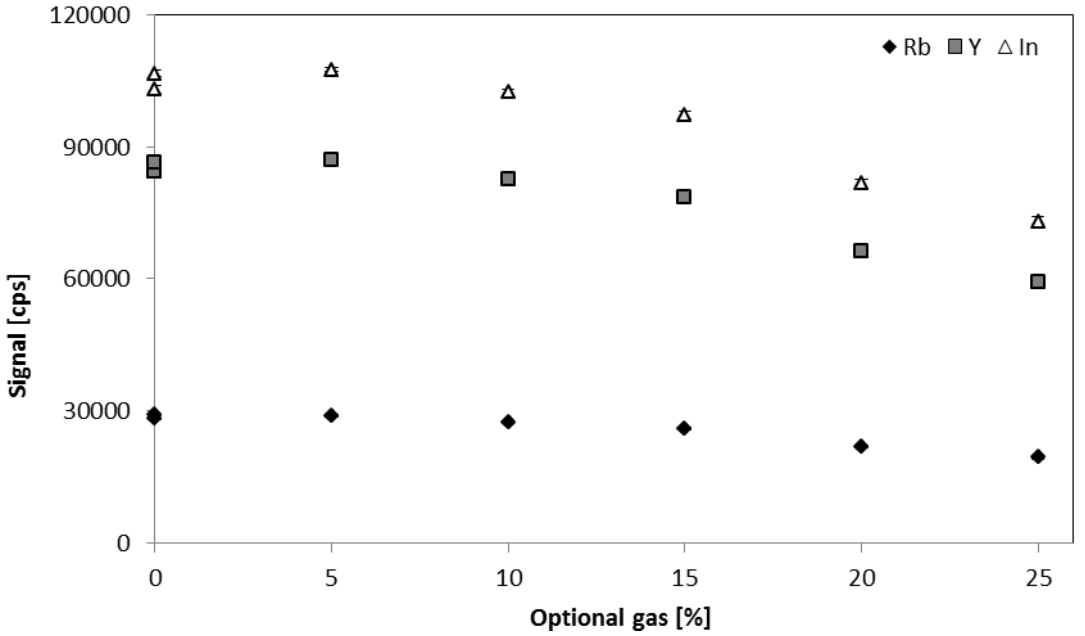


Figure 27 Signal intensities of Rb, Y and In, used as internal standard in a 20 mM ammonium formate buffer matrix, depending on the amount of optional gas (1 % CO_2 in Ar (v/v)), measured with ICPMS, standard deviations too small to see

4.1.3 Selection of an adequate mobile phase for cation-exchange chromatography

The major difference of the three used mobile phases was the retention time of AB (see Figure 28 and Figure 29). AB retained much longer on the column with the two mobile phases containing ammonium formate than with pyridine. This could be improved by increasing the ammonium formate concentration in the buffer. The 0.1 µg/L AB standard could be detected hardly with all three mobile phases (see Figure 29).

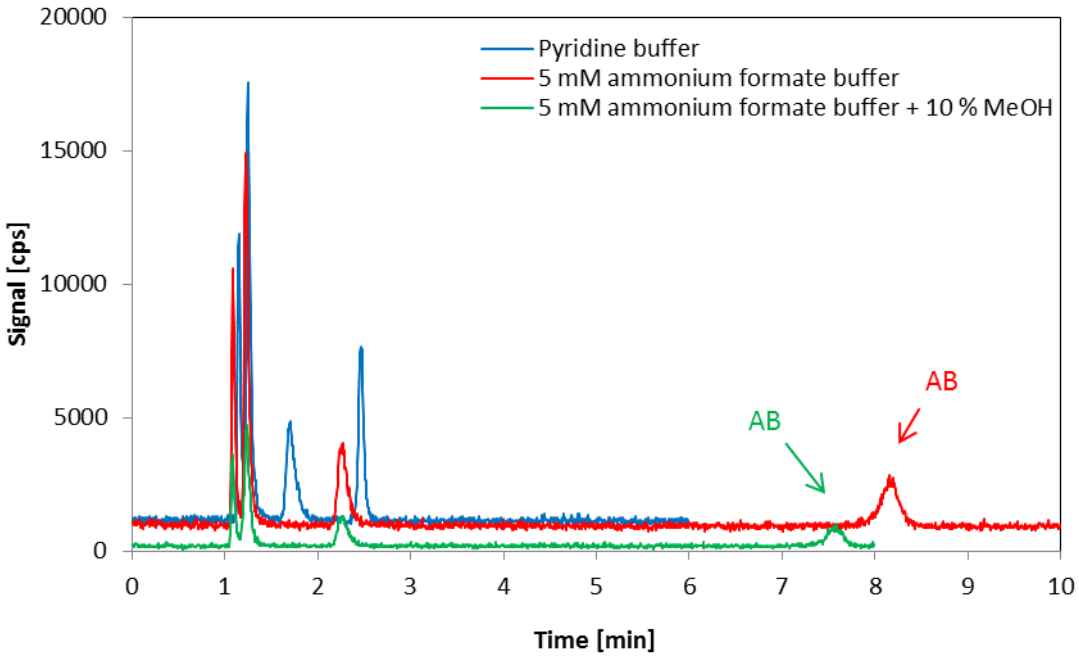


Figure 28 DMA, MA, AB, As(III) and As(V) at a concentration of 1 µg/L, measured with different mobile phases with HPLC-ICPMS on a Zorbax 300-SCX column, injection volume 20 µL

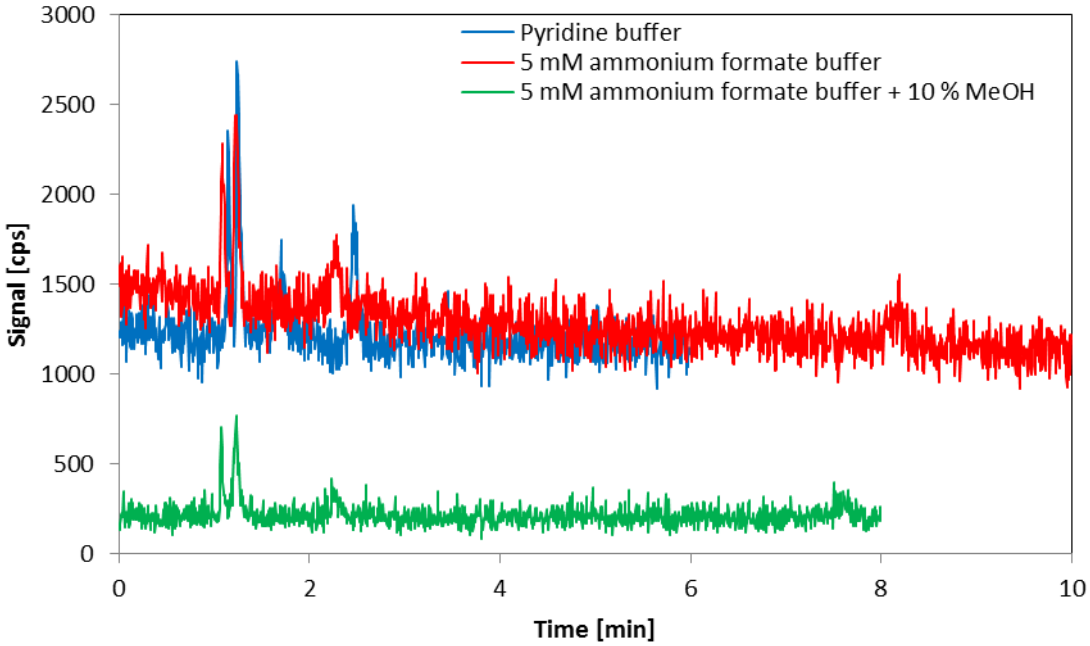


Figure 29 DMA, MA, AB, As(III) and As(V), at a concentration of 0.1 µg/L, measured with different mobile phases with HPLC-ICPMS on a Zorbax 300-SCX column, injection volume 20 µL

Retention of several cations

Urine sample 32307, containing ~210 µg/L AB, was measured with HPLC-ICPMS to be sure that Na, K, Ca and Mg do not co-elute with AB. A chromatogram is shown in Figure 30.

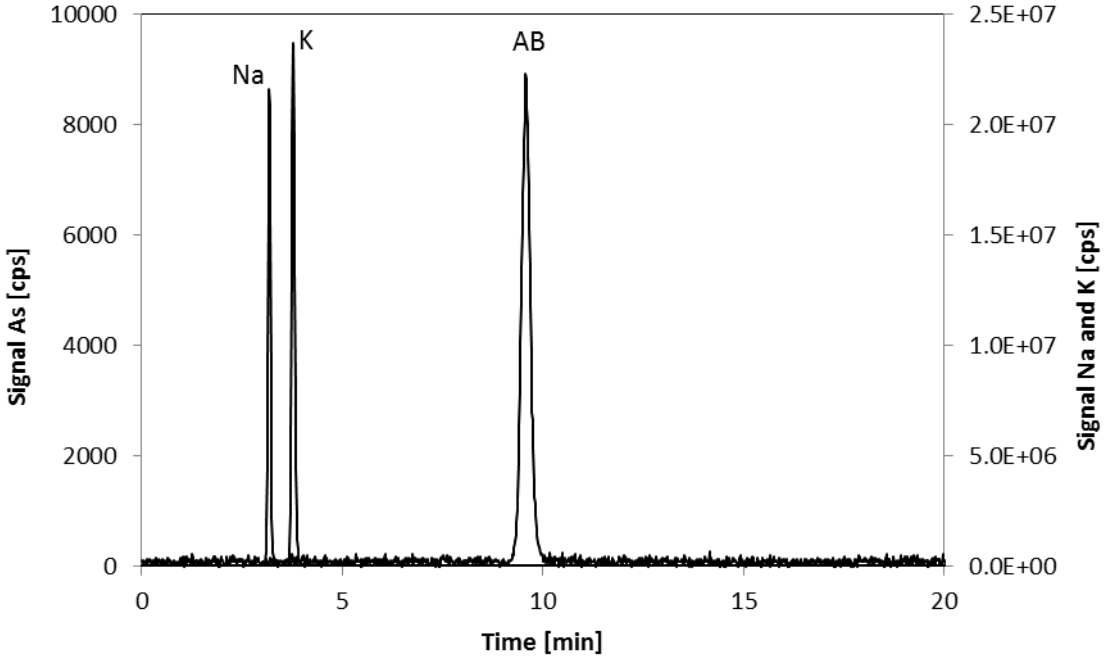


Figure 30 Retention of Na, K and AB in urine sample 32307 measured with HPLC-ICPMS with a 20 mM ammonium formate buffer (pH 2.5) as mobile phase on a Zorbax 300-SCX column

As shown in Figure 30 the singly charged cations Na^+ and K^+ elute before AB and the doubly charged cations Mg^{2+} and Ca^{2+} do not elute in a runtime of 20 minutes. Therefore, Na^+ and K^+ should not lead to any signal suppression in ESMS under these conditions. The two doubly charged cations, Mg^{2+} and Ca^{2+}, may lead to signal suppression because they will elute later and maybe at the same time as AB in a further sample.

4.1.4 Optimization of the MeOH content in the mobile phase for HPLC-ESMS

A urine sample, spiked with 80 µg/L DMA, 10 µg/L MA, 100 µg/L AB, 5 µg/L As(III) and 5 µg/L As(V) was measured with a 20 mM ammonium formate buffer (pH 2.5) and different amounts of MeOH as mobile phase with HPLC-ESMS.

M/z = 179 was extracted from the SIM and integrated. The retention times and the areas of AB, measured with different mobile phases, were compared. Furthermore the AB peak was overlaid with the scan to observe if any matrix compounds elute at the same time. The integration results can be found in Figure 31.

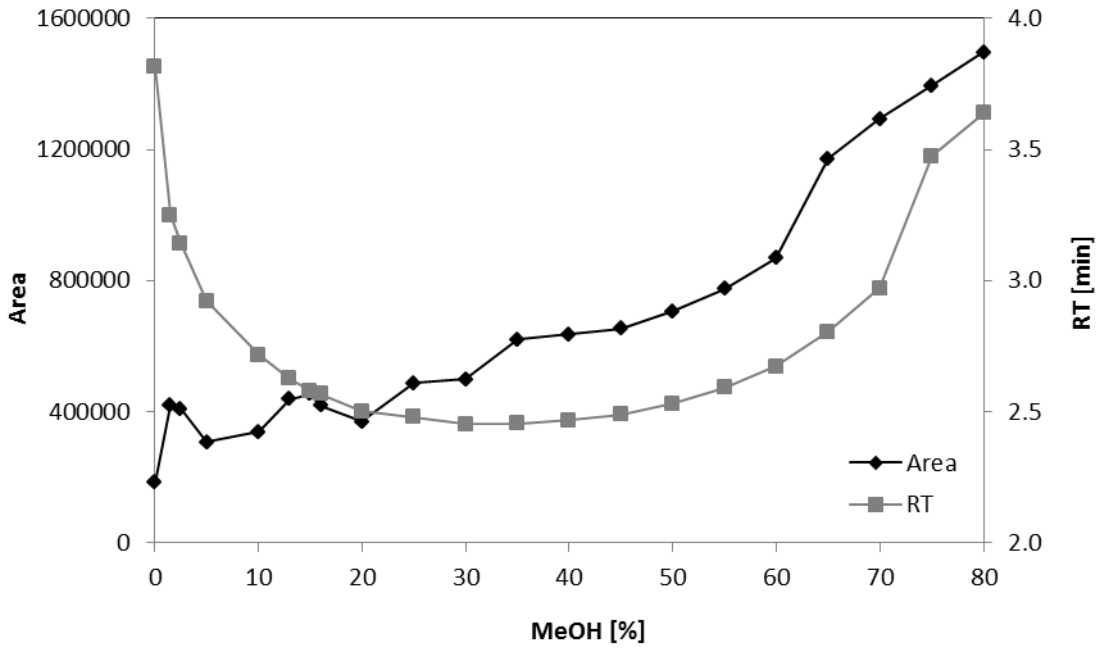


Figure 31 Area and retention time (RT) of AB, measured with HPLC-ESMS, depending on the MeOH content; mobile phase: 20 mM ammonium formate buffer + different amounts of MeOH

Figure 31 shows a big increase of the area with increasing amount of MeOH. The retention time firstly decreases and increases with 30 % MeOH again. A maximal area and minimal retention time would be preferable. For this reason 65 % of MeOH seemed to be ideal.

Because coelution of AB with matrix compounds would not be desirable, the matrix from m/z = 70 to 300, measured in the scan mode, was overlaid with the signal of AB extracted from the SIM. This was done for each methanol content shown in Figure 31. With only 2.5 % of MeOH, AB overlaid with some matrix compounds and with 80 % of MeOH the matrix background at the retention time of AB increased again (see Figure 32 to Figure 34). This is the reason why a 20 mM ammonium formate buffer (pH 2.5) with 65 % of MeOH seemed to be the mobile phase of choice.

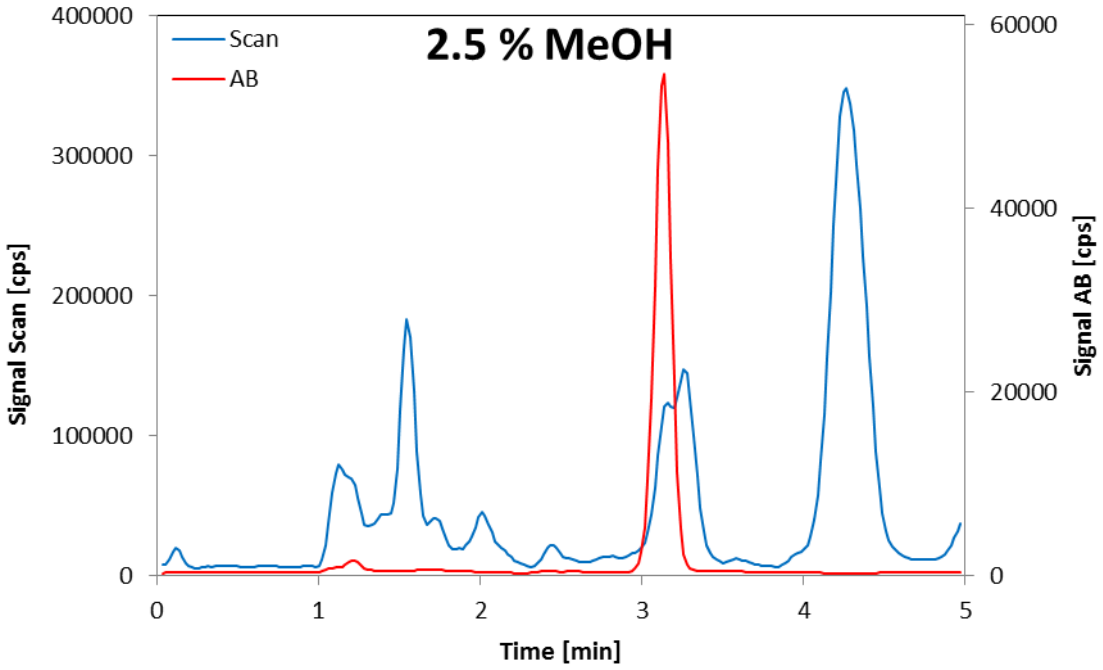


Figure 32 Chromatogram of urine spiked with 100 µg/L AB measured with HPLC-ESMS, 20 mM ammonium formate buffer pH 2.5 and 2.5 % MeOH as mobile phase, AB = m/z 179 extracted from SIM

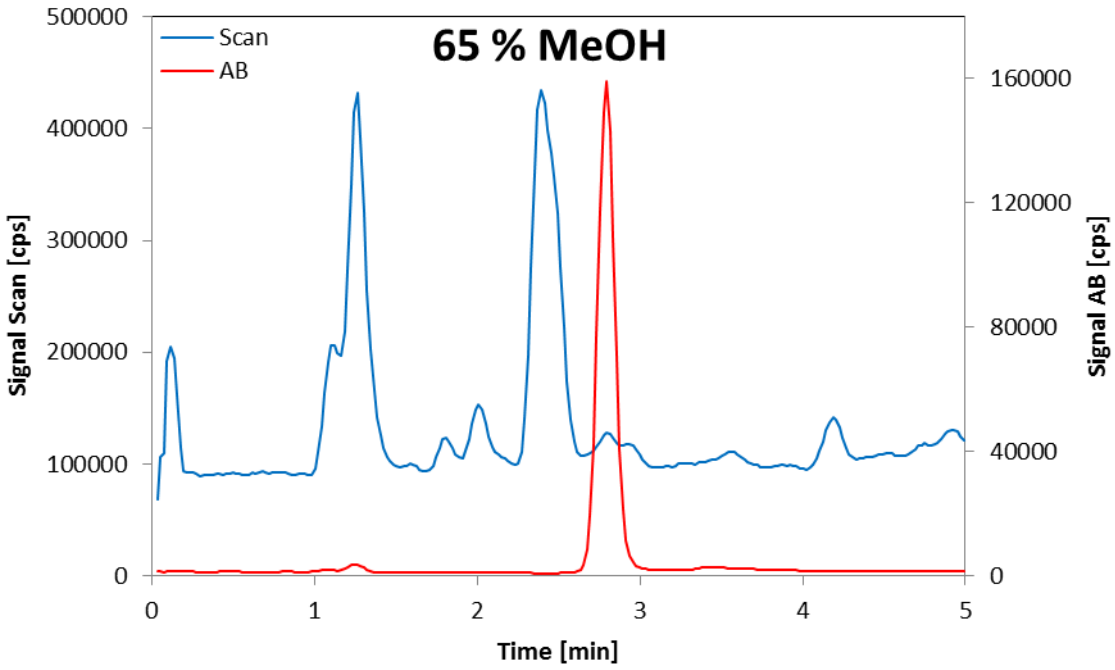


Figure 33 Chromatogram of urine spiked with 100 µg/L AB measured with HPLC-ESMS, 20 mM ammonium formate buffer pH 2.5 and 65 % MeOH as mobile phase, AB = m/z 179 extracted from SIM

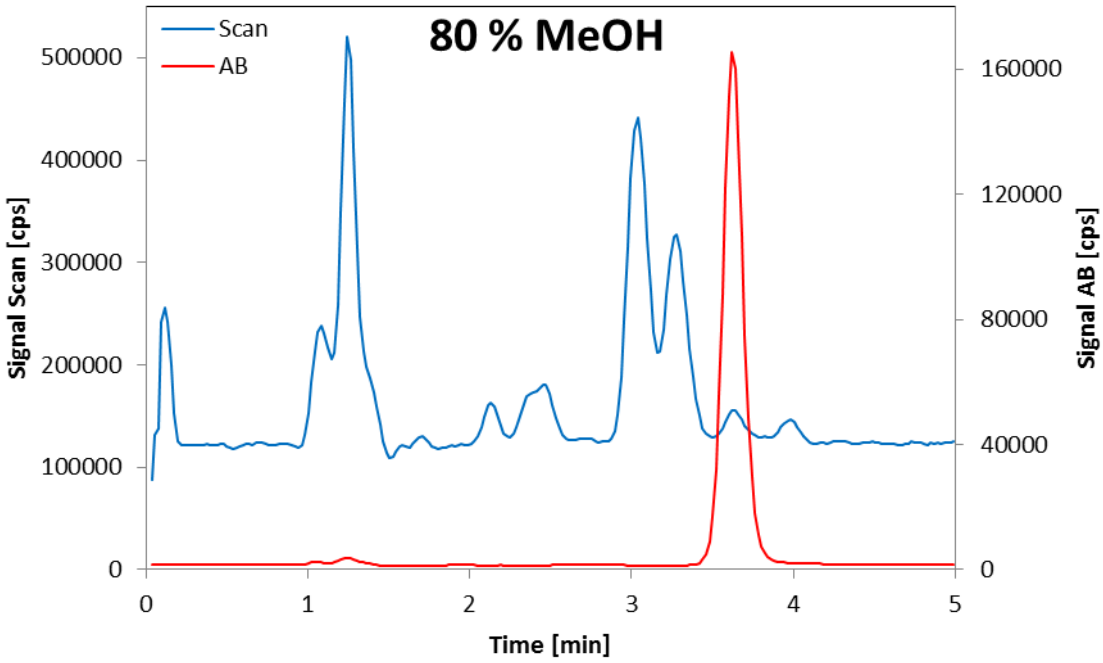


Figure 34 Chromatogram of urine spiked with 100 µg/L AB measured with HPLC-ESMS, 20 mM ammonium formate buffer pH 2.5 and 80 % MeOH as mobile phase, AB = m/z 179 extracted from SIM

4.2 USG - a good way of normalisation?

Two urine samples (12204 and 42204) with high USG (1.022 and 1.024) and a control urine sample from March 2015 (150317) with low USG (1.008) were diluted, to proof the linear correlation between the USG and the water content of the urine sample. The five replicates of each dilution step showed always the same USG. Because of this only one dot is presented in Figure 35 for each dilution step of the urine sample.

The USG and the dilution of the urine correlate quite well especially at the urine samples with high USG (see Figure 35). The lower the USG the worse the correlation, perfectly seen at sample 150317. This results show that a normalisation of the urine samples by USG might lead to mistakes when dealing with urine samples with low USG. Because no better method to normalise urine samples is available we still used the normalisation with USG to make our results comparable.

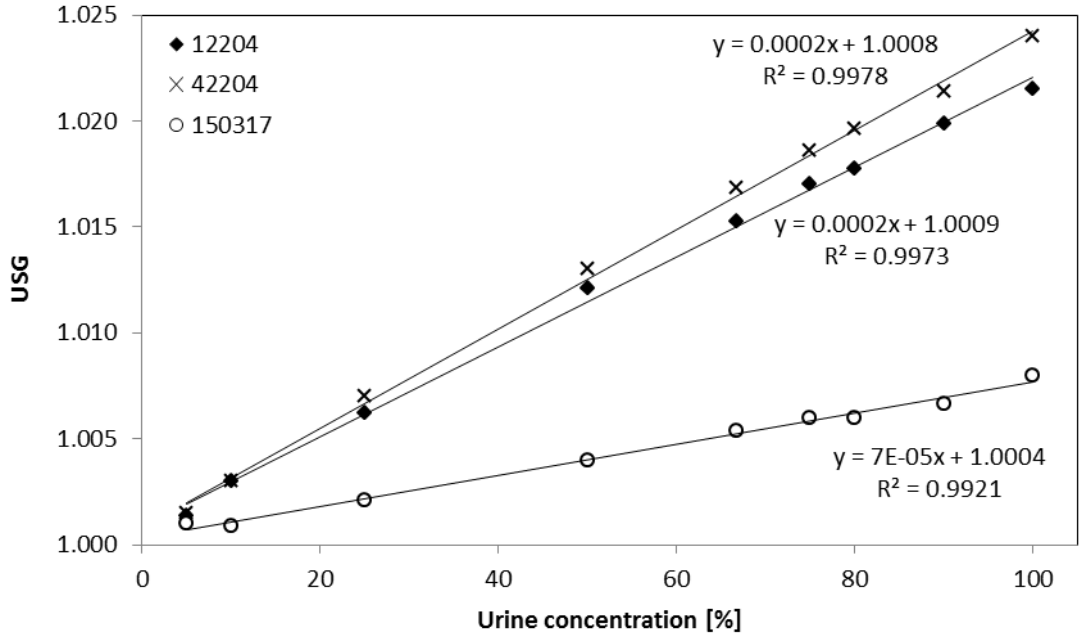


Figure 35 Correlation of the USG and the dilution of the urine samples 12204, 42204 and 150317 (not from the study), measured with the total solids refractometer, 100 % = not diluted urine, 50 % = urine : water = 1 : 1

4.3 Determination of Total Element Concentrations

The urine specific gravity (USG) of all urine samples was determined. The ranges for all samples together and for all 5 volunteers can be found in Table 20.

Table 20 USG of the urine samples compared with the total amount of excreted urine

	All	Person 1	Person 2	Person 3	Person 4	Person 5
Range	1.002-1.042	1.002-1.028	1.003-1.042	1.002-1.012	1.005-1.026	1.002-1.024
Median	1.011	1.015	1.013	1.005	1.017	1.018
Amount of urine [L][1]	8.0[2]	8.4	6.3	16.6	3.3	5.6
Urine/day [L]	2.3	2.4	1.8	4.7	0.9	1.6

[1] total amount of urine over 3.5 days; [2] mean amount of urine from all 5 volunteers

The urine samples from person 2 show a higher range of USG but the median is nearly the same for person 1, 2, 4 and 5 (Table 20). Person 3 shows a lower specific gravity than the others. This is because person 3 urinated much more than the other 4 volunteers.

4.3.1 Elemental composition of different urine samples

The concentrations of 29 elements in the urine samples were determined. For some elements the measured concentrations of the 5 volunteers were quite similar and for others big differences could be observed. For this reason the concentration range and the median were calculated for all 5 volunteers together and for each person itself. The concentrations were normalised by USG (see Equation 2) and can be found in Table 21. The values for arsenic were excluded, because after the ingestion of 2 mg As, they were not representative for normal urine.

As seen in Table 21 the element concentrations of person 3 are much lower than the concentrations of the other volunteers. This is because of the high amount of urine that person 3 excreted.

Because the amount of urine was very different between the 5 volunteers, it was interesting to sum up the total amount of each element urinated from each person. Therefore, the total amount of each element in each urine sample was calculated and all samples from one person were summed up. To simplify comparison the summed values were divided by 3.5 to get the amount of each element excreted per day. The results are shown in Figure 36 to Figure 39.

Table 21 Range and median of different elements in the urine samples, normalised by USG, measured with ICPMS

		All		Person 1		Person 2		Person 3		Person 4		Person 5	
		Range	Median	Range	Median	Range	Median	Range	Median	Range	Median	Range	Median
Li	[µg/L]	5.2-420	20	30-410	69	7.9-310	23	5.2-280	14	8.1-300	12	9.4-420	25
B	[µg/L]	230-1700	600	460-1200	730	230-12	580	240-680	340	440-1400	660	380-1700	790
Na	[mg/L]	300-5200	1500	1000-2900	1700	970-3800	1700	300-1200	640	410-5200	2000	1500-4800	2200
Mg	[mg/L]	11-140	57	49-140	78	30-140	92	11-35	20	42-130	68	12-88	39
P	[mg/L]	11-1500	440	280-1500	820	11-1200	360	82-370	190	190-1500	550	420-1500	880
S	[mg/L]	100-870	500	450-830	570	220-620	500	100-280	190	370-870	550	480-870	590
K	[mg/L]	170-5200	1200	790-3100	2200	320-2900	1200	170-1800	730	510-2700	1100	560-5200	1400
Ca	[mg/L]	11-300	92	49-160	87	24-220	94	11-52	27	110-310	180	41-230	150
V	[µg/L]	0.1-1.8	0.7	0.4-1.8	0.9	0.4-1.3	0.8	0.1-0.7	0.3	0.4-1.3	0.7	0.6-1.4	0.8
Cr	[µg/L]	<0.5-4.4	0.7	<0.5-4.4	0.9	<0.5-2.7	1.1	<0.5-3.3	<0.5	<0.5-1.8	0.6	<0.5-3.6	1.0
Mn	[µg/L]	<0.1-8.7	0.6	0.3-4.5	0.6	0.3-8.7	0.8	<0.1-3.0	0.2	0.1-2.5	0.4	0.7-8.2	2.0
Fe	[µg/L]	2.1-180	21	13-110	21	7.8-140	36	2.1-120	8.8	9.3-96	17	10-180	40
Co	[µg/L]	<0.2-1.2	0.3	0.2-0.4	0.3	0.2-0.6	0.3	<0.2-0.5	0.2	0.2-1.2	0.5	0.2-0.5	0.3
Ni	[µg/L]	<1.0-43	2.6	0.4-14	2.5	1.4-19	2.9	<1.0-43	1.0	2.5-24	3.8	*	
Cu	[µg/L]	2.3-170	11	8.9-54	14	6.1-27	11	2.3-140	4.7	10-75	14	8.0-170	11
Zn	[µg/L]	12-940	210	230-670	350	44-390	150	12-360	32	88-530	300	180-940	300
Se	[µg/L]	7.5-62	26	23-50	31	10-29	24	7.5-18	11	23-43	33	25-62	30
Rb	[mg/L]	0.1-3.5	1.0	0.9-3.1	2.4	0.3-1.9	1.0	0.1-1.2	0.6	0.4-2.0	0.9	0.6-3.5	1.3
Sr	[µg/L]	35-500	150	130-500	250	51-280	150	35-110	75	140-310	200	83-240	150
Mo	[µg/L]	1.9-130	26	16-130	36	18-83	41	1.9-19	5.7	17-94	37	7.8-79	22
Cd	[µg/L]	<0.1-1.0	0.2	0.2-0.8	0.5	0.1-0.4	0.2	<0.1-1.0	<0.1	<0.1-1.0	0.1	<0.1-0.5	0.2
Sb	[µg/L]	<0.1-0.5	0.1	<0.1-0.2	0.1	<0.1-0.5	0.1	<0.1-0.5	<0.1	<0.1-0.2	0.1	<0.1-0.4	0.1
Te	[µg/L]	<0.2-0.8	0.2	<0.2-0.7	0.2	<0.2-0.8	<0.2	<0.2-0.8	<0.2	<0.2-0.8	0.2	<0.2-0.5	0.2
Ba	[µg/L]	0.5-29	3.6	2.6-29	4.5	3.2-18	6.7	0.5-17	1.7	2.3-18	3.5	2.6-7.4	3.5
Hg	[µg/L]	0.2-4.5	1.0	0.7-3.5	1.2	0.5-3.5	1.6	0.2-2.0	0.5	0.3-2.5	0.5	1.5-4.5	2.8
Pb	[µg/L]	<1.0-14	1.4	1.1-10	1.5	1-2-7.7	2.7	<1.0-14	1.3	<1.0-9.8	1.1	<1.0-11	1.0
Bi	[µg/L]	<0.1-2.0	<0.1	<0.1-0.3	<0.1	<0.1-0.7	<0.1	<0.1-2.0	<0.1	<0.1-0.5	<0.1	<0.1-0.6	<0.1
U	[µg/L]	<0.1-0.1	<0.1	<0.1-0.1	<0.1	<0.1-0.1	<0.1	<0.1-0.1	<0.1	<0.1-0.1	<0.1	<0.1-0.1	<0.1

***** could not be quantified

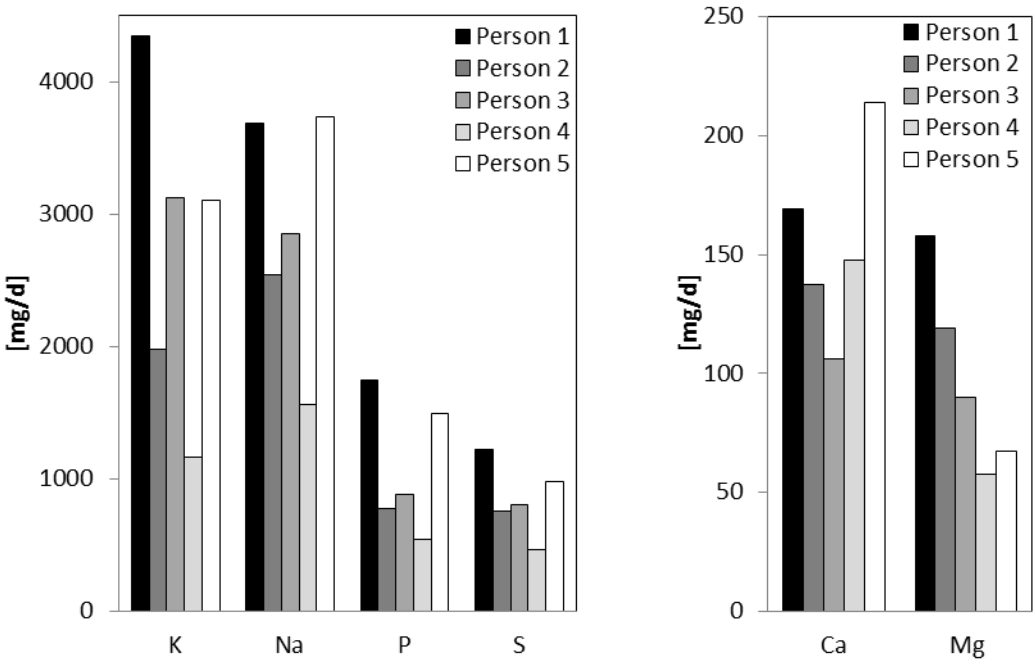


Figure 36 Total amount of excreted K, Na, P, S, Ca and Mg in urine per day, measured with ICPMS

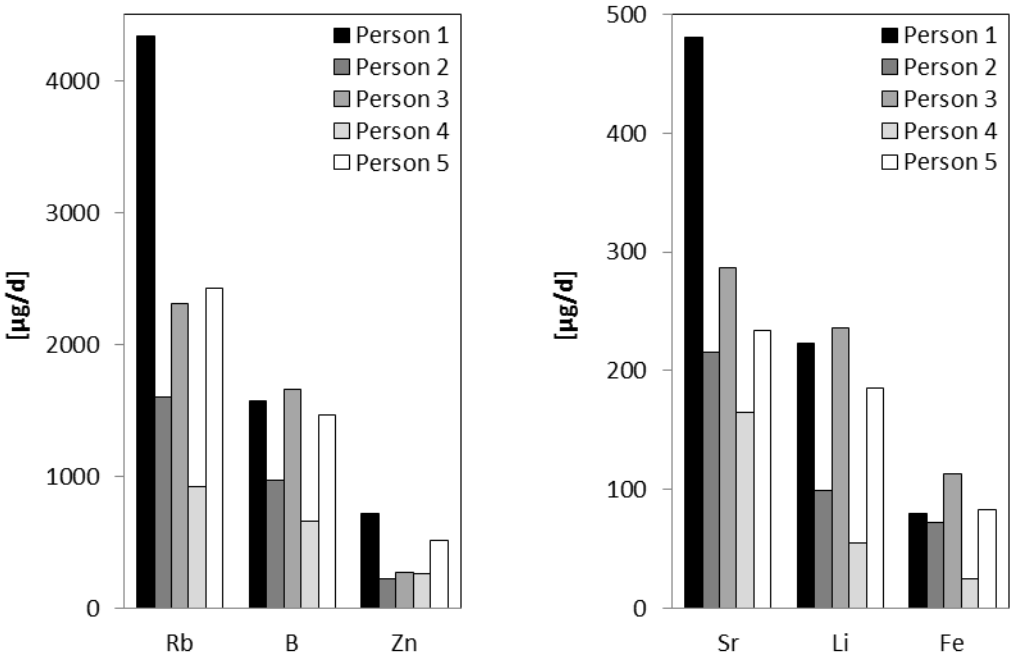


Figure 37 Total amount of excreted Ni, Rb, B, Zn, Sr, Li and Fe in urine per day, measured with ICPMS

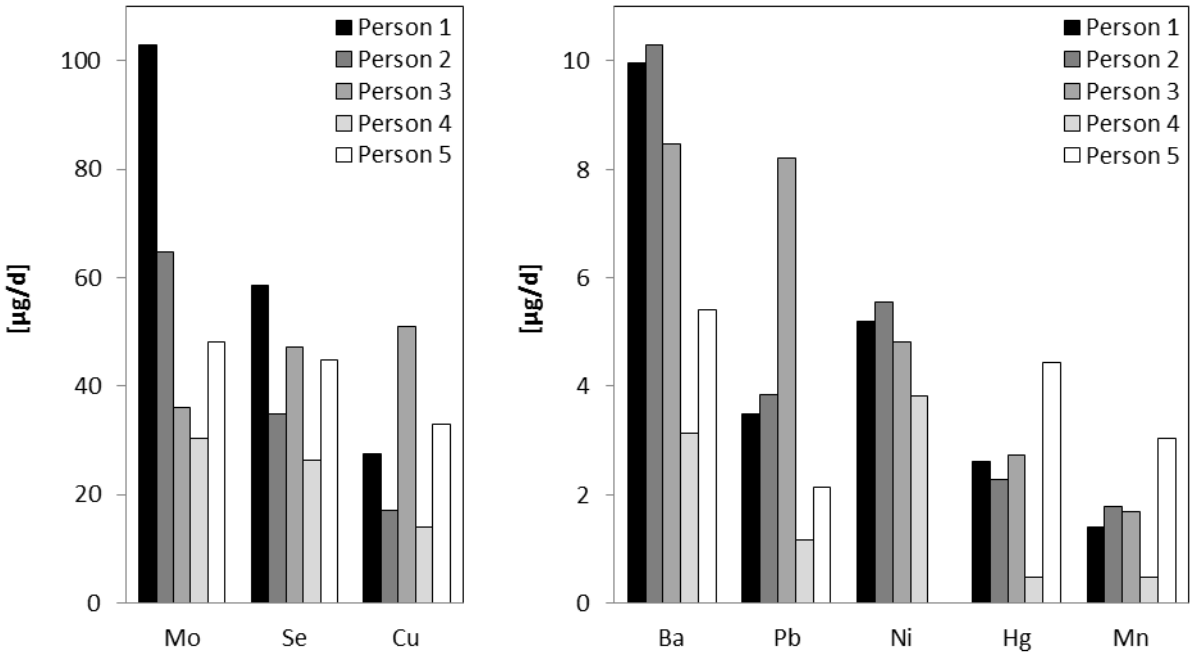


Figure 38 Total amount of excreted Mo, Se, Cu, Ba, Pb, Ni, Hg and Mn in urine per day, measured with ICPMS

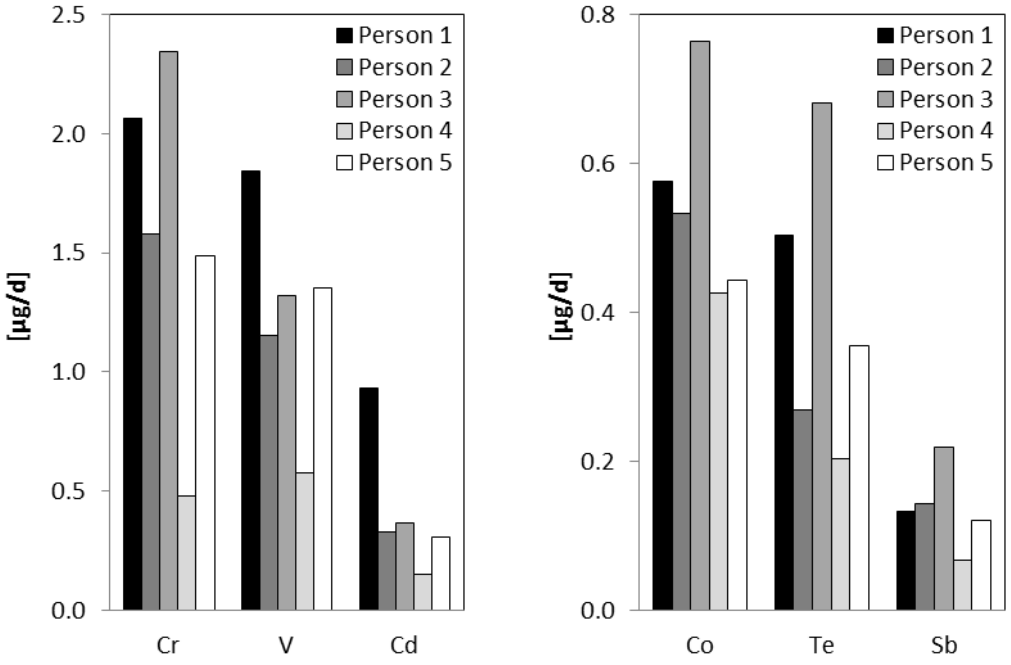


Figure 39 Total amount of excreted Cr, V, Cd, Co, Te, Bi and Sb in urine per day, measured with ICPMS

As shown in Figure 36 to Figure 39 the 5 volunteers are quite different. Person 3, which showed relatively low elemental concentrations in the urine samples, does not show low amounts of total excreted elements. Due to the high amounts of excreted urine (~4.7 L/d), even low elemental concentrations can end up in high amounts excreted in total per day. Of course person 3 had to drink a lot to generate these high amounts of urine, and of course there are elements in the drinking water as well.

Person 4, who has excreted lowermost amount of urine (~0.9 L/d), showed the lowermost amount of nearly all elements. Comparing this with the high amounts of excreted elements of person 3, drinking water seemed to be an important source for many elements.

Of course not only the drinking behaviour but the eating behaviour has a big influence on the elemental composition in the urine sample. For example person 1 excreted high amounts of K, Na, Rb, Sr, Mo and V whereas person 3 excreted high amounts of Fe, Cu, Pb, Co, Te and Sb.

To check our results, we compared them with literature values. In literature commonly the elemental concentrations or the total excreted amounts per day were evaluated. Both are shown in Table 22.

Table 22 Comparison of our study with references: Comparison of elemental concentrations in urine and the total amount of different elements in urine per day; Used References for the concentration values: [93-101]; Used references for the total amount per day: [60, 101-106]

	Study	**Reference**	**Study**	**Reference**
	[mg/L]		[mg/day]	
Na	300-5200	-	1600-3700	1600-3900[1]
Mg	11-140	-	57-160	60-210[2]
K	170-5200	-	1200-4300	1300-3300
Ca	11-300	-	110-210	110-320
	[µg/L]		[µg/day]	
Li	5.2-420	4-237	55-240	25-100
B	230-1700	282-2072	660-1700	500-3000
V	0.1-1.8	0.1-10	0.6-1.8	0.2-1
Cr	<0.5-4.4	0.04-3.5	0.5-2.3	0.2-2
Mn	<0.1-8.7	<0.1-7.8	0.5-3	0.5-2
Fe	2.1-180	-	25-110	100-200
Co	<0.2-1.2	<0.2-10	0.4-0.8	0.5-2
Ni	<1.0-43	<0.1-59	3.8-5.5	2-8
Cu	2.3-170	1-80	14-51	30-60
Zn	12-940	8-970	230-720	400-600
Se	7.5-62	4-180	26-59	25-50
Rb	100-3500	60-4100	900-4300	1000-4000
Sr	35-500	11-680	160-480	110-390
Mo	1.9-130	2.8-357	30-100	20-30
Cd	<0.1-1.0	<0.1-7.4	0.1-0.9	1-5
Sb	<0.1-0.5	<0.1-0.6	0.1-0.2	<0.1-5
Te	<0.2-0.8	0.1-0.5	0.2-0.7	-
Hg	0.2-4.5	0.1-10	0.5-4.4	5-20
Pb	<1.0-14	0.5-30	1.2-8.2	10-20

[1] 5900 mg/day according to [60]; [2] 400 mg/day according to [60]

The results from our study fit quite well with the literature values. Only some values are a bit lower or higher than the reported ones.

4.3.2 Correlations of element concentrations in human urine

To verify correlations between certain elements in human urine linear correlations were calculated for all elements (correlation coefficient *r*). This was done for each person separately and additionally for all five candidates together. To exclude correlations caused by dilution, the normalised concentrations of each element were used. Furthermore the correlations between USG and the concentrations (without normalisation) were calculated. Some examples are shown below.

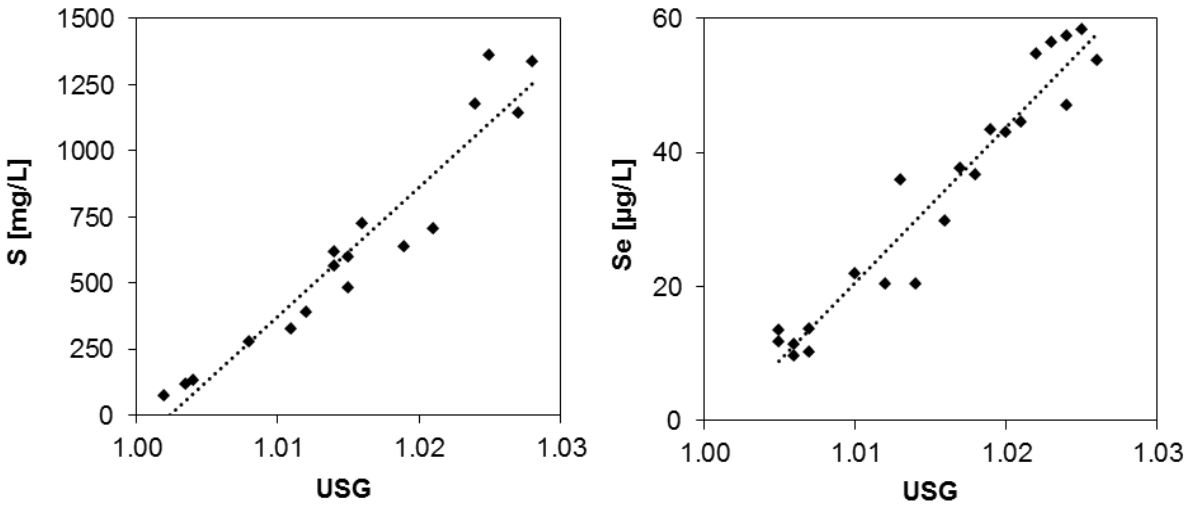


Figure 40 Strong correlations of S and the USG in the urine samples of person 1 (r = 0.96) and of Se and the USG in the urine samples of person 4 (r = 0.97), concentration not normalised and measured with ICPMS, USG measured with the total solids refractometer

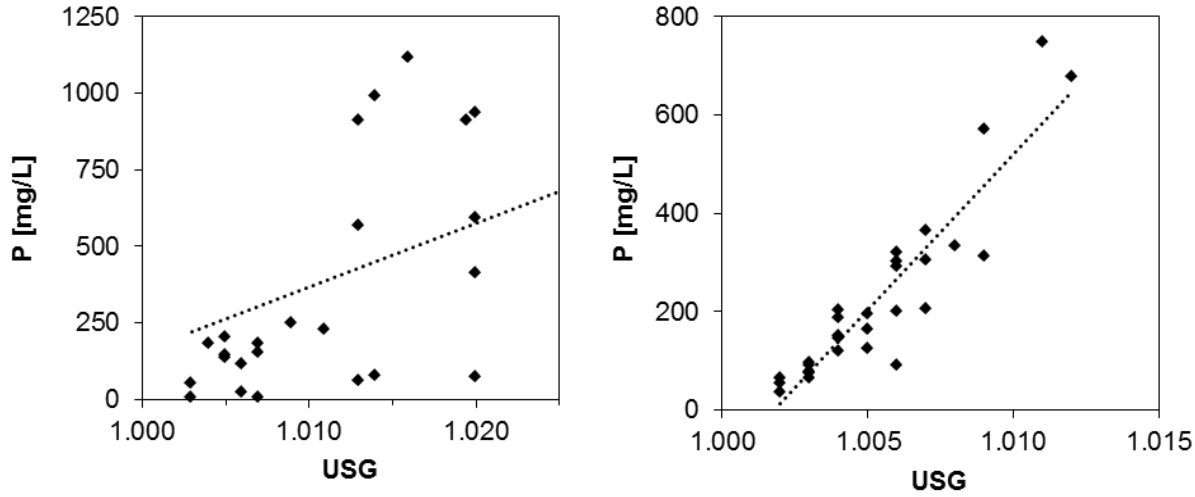


Figure 41 Correlations of the USG and P, no correlation in the urine samples of person 2 (r = 0.39) and a strong correlation in the urine samples of person 3 (r = 0.92), concentrations not normalised and measured with ICPMS, USG measured with the total solids refractometer

S, V, Se and Sr highly correlated with the USG ($r > 0.7$) in the samples of all 5 candidates (see Figure 40). On the other hand Cr, Mn and Te showed no correlations ($r < 0.4$), neither for each person individually, nor for all 5 together. The correlations of some elements like Na, P, K, Ca and Rb showed an interesting behaviour; for 4 of the 5 volunteers the correlations of those elements with the USG were high ($r > 0.7$) and one person (not always the same) had nearly no correlations ($0.38 < r < 0.59$). For all 5 together, high correlations could be observed again (see Figure 41).

When looking at the correlation coefficients of all the elements in the normalised urine samples of all 5 volunteers together and everyone separately, big differences could be observed. This is of course again because of their different drinking and eating behaviour.

Some examples for strong correlations found in the urine samples of every person and all 5 volunteers together are listed in Table 23 and two examples are shown in Figure 42.

Table 23 Strong correlations between different elements in human urine, normalised by USG, measured with ICPMS

Correlation	*r*	Correlation	*r*	Correlation	*r*
K - Rb	> 0.93	Mg - Sr	> 0.8	Sr - Ca	> 0.7
Ni - Cu	> 0.91*	Li - B	> 0.7		

***** person 2: $r = 0.64$

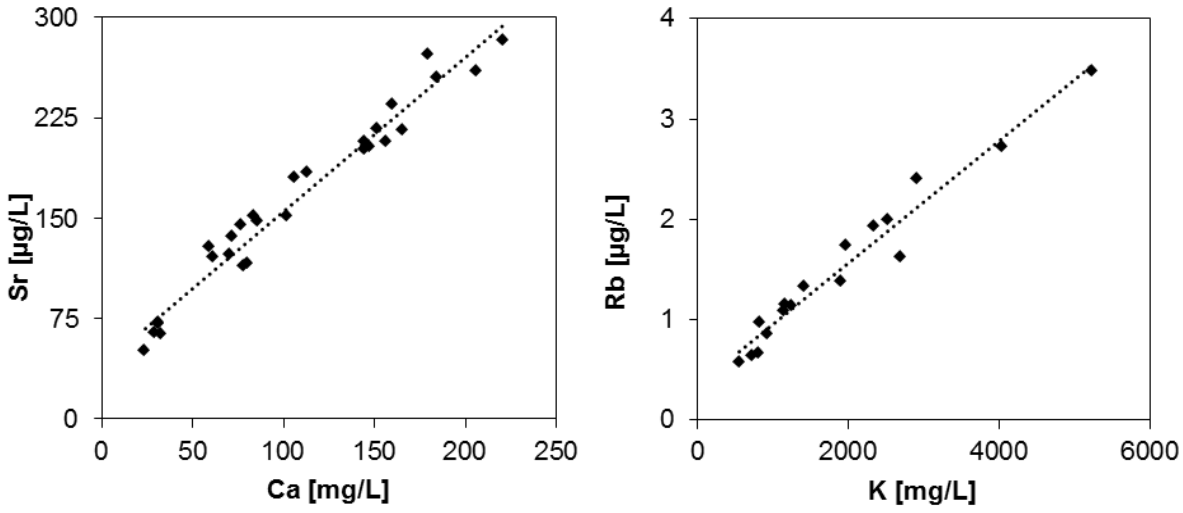


Figure 42 Strong correlations between Ca and Sr in the urine samples of person 2 ($r = 0.98$) and K and Rb in the urine samples of person 5 ($r = 0.98$), concentrations normalised by USG, measured with ICPMS

Beside positive correlations, negative ones could be observed as well. Sodium, the element with the highest concentrations in the urine samples, showed quite interesting correlations. Strong correlations between Na and V could be observed for person 1, 4 and 5 ($0.69 < r < 0.8$) but no further positive correlations for Na could be observed. However, person 4 showed strong negative correlations between Na and S ($r = 0.67$), Na and Zn ($r = 0.60$) and Na and Se ($r = 0.75$) (see Figure 43). No other person showed mentionable correlations between these elements. The reason for those high negative correlations of person 4, the person with the lowest amount of urine, are not investigated at this point.

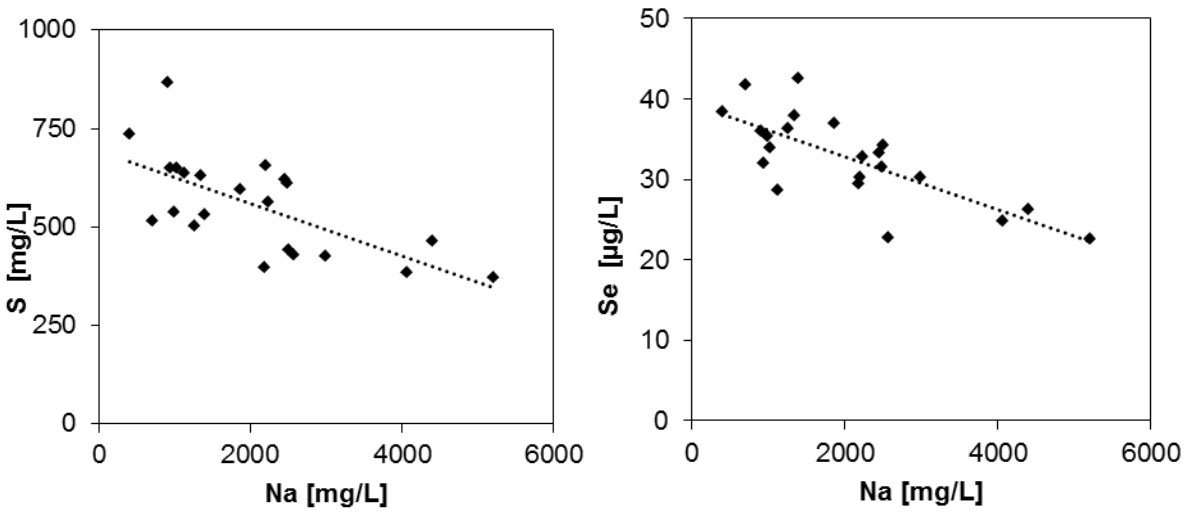


Figure 43 Negative correlations between Na and S (r = 0.67) and Na and Se (r = 0.75) in the urine samples of person 4, concentrations normalised by USG, measured with ICPMS

4.3.3 Element-ranking in human urine

Especially for the measurements with ESMS the ranking of the different element was of interest. Therefore, the different elements were ranked after their concentration in each urine sample. The ranking list did not vary a lot between the different volunteers and all samples.

Na was nearly always the element with the highest concentration in human urine. Sometimes it was the element with the second highest concentration and only once it was at rank 4 (the element with the fourth highest concentration).

Other elements which were present in high concentrations in the urine samples are:

K	always in the top 5 (the 5 elements with the highest concentrations)
P	nearly always in the top 5, always in the top 6
S	always in the top 5
Mg	nearly always at rank 5, sometimes at rank 4 or rank 6
Ca	nearly always at rank 6, sometimes at rank 5

The elements were mainly ingested via the diet. The major source of sodium is cooking salt (NaCl) [107]. Besides this some vegetables like meadow mushrooms (43 g/kg dry mass), spinach (8.2 g/kg dry mass) and red radish (4.6 g/kg dry mass) as well as marine fish (6.0-29 g/kg dry mass) provide high amounts of sodium [108].

Foods with a high potassium content are for example soybeans (17 g/kg dry mass), chanterelle (5.1 g/kg dry mass), potatoes (4.4 g/kg dry mass), bananas (3.9 g/kg dry mass) and milk (1.5 g/kg dry mass) [107, 109].

Phosphorus is mainly found in meat, milk and their products [109].

Foods high in sulphur are meat (~2 g/kg wet mass), eggs (2 g/kg wet mass), fish (1.3-2.4 g/kg wet mass) and potatoes (1.7 g/kg wet mass) [108].

Magnesium is mainly found in green vegetables (for example spinach or avocados) seeds, nuts and beans [110].

One of the best calcium providers are milk, milk products, nuts and legumes [107, 109] (for example broccoli [108]). Some fruits like kiwis and oranges are high in calcium as well [108].

4.4 Arsenic Speciation Analysis

Arsenic speciation analysis was done with all urine samples with cation-exchange and anion-exchange chromatography focusing on As(III), As(V), AB, DMA and MA. Neither with anion-exchange chromatography nor with cation-exchange chromatography unknown arsenicals could be observed.

In cation-exchange chromatography the first peak (As(III)) and the second peak (As(V) and MA) were integrated together, because they were not baseline separated in many samples. DMA and AB were baseline separated.

In anion-exchange chromatography all 4 peaks were integrated separately; cationic As, DMA, MA and inorganic As. Because of the previous cation-exchange chromatography, the cationic As was assumed to consist only of AB.

The comparison of the two chromatographic methods showed a good mass balance (after ingestion of d^9AB always between 90 and 110 % for AB) and when integrating the whole chromatograms the results were good comparable with the total amount of arsenic, measured with ICPMS (mass balance between 90 and 110 %).

Before the ingestion of d^9AB different arsenic species (AB, DMA, MA and inorganic As) were present in low amounts in the urine samples. After the ingestion of d^9AB more than 96 % of the measured arsenic was AB or cationic arsenic. The results of the speciation analysis can be found in Table 37 in the appendix.

4.5 Urine Matrix and ESMS

4.5.1 Signal suppression caused by the urine matrix

To get an impression of the signal suppression in ESMS due to the urine matrix some spike experiments were performed. Urine sample 12204 with a USG = 1.021 was spiked with different amounts of AB. Furthermore urine sample 12204 spiked with ultrapure water and ultrapure water spiked with AB were measured for comparison.

The procedure was always the same; un-spiked urine was measured 3 times, afterwards 3 times urine spiked with a certain concentration of AB followed by 3 un-spiked samples and the next concentration were measured. The three replicates of the un-spiked urine samples and all the spiked urine samples were similar. For this reason only one run of each concentration of AB is shown in Figure 44.

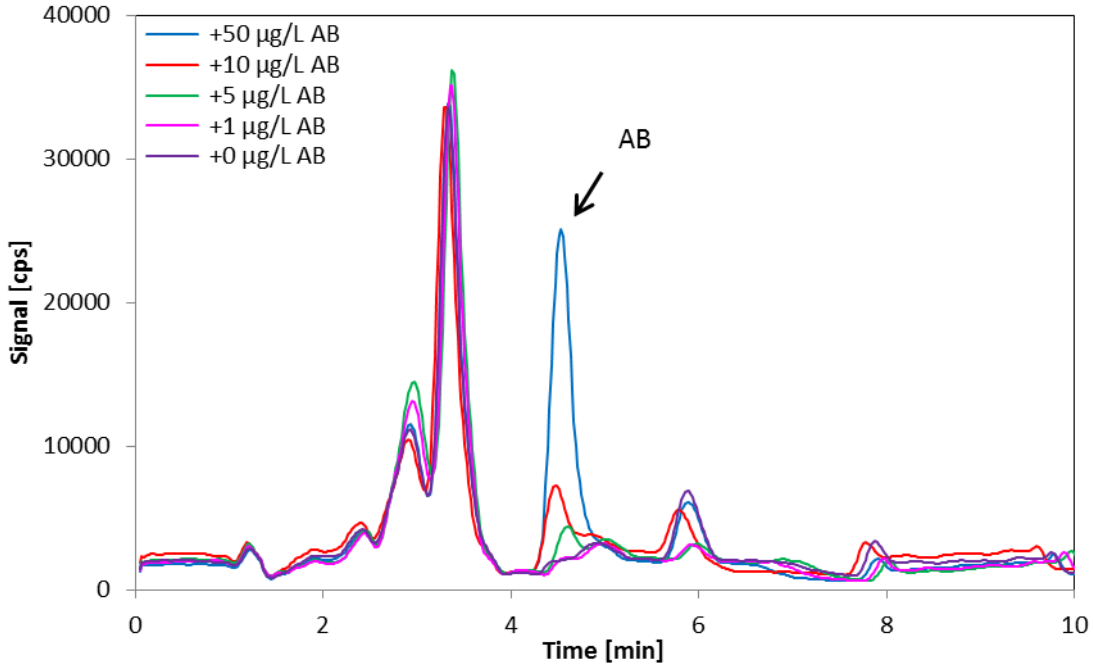


Figure 44 Chromatogram of urine sample 12204 (USG = 1.021), spiked with different amounts of AB, measured with HPLC-ESMS

Urine spiked with 50 µg/L AB led to a high peak of AB as shown in the chromatogram above (Figure 44). The lower concentrations 10 µg/L and 5 µg/L AB added to the urine, could be observed as well. For concentrations < 5 µg/L AB the peak could not be distinguished from the urine matrix any more.

Further experiments have shown that the signal suppression of the urine matrix depends strongly on the USG. Lower USG, this means less concentrated urine, lead to lower signal

suppression. Therefore, it is not possible to quantify AB in urine samples with different USG. If quantification is not necessary we can detect AB up to ~5 µg/L, in rather concentrated urine, with our method.

Influence of the USG on the signal suppression caused by the urine matrix in ESMS

Urine sample 12204 was diluted stepwise, spiked with 10 µg/L d^9AB and measured with HPLC-ESMS. The USG of each sample was measured and can be found, as well as the integration results, in Figure 45 and Table 24.

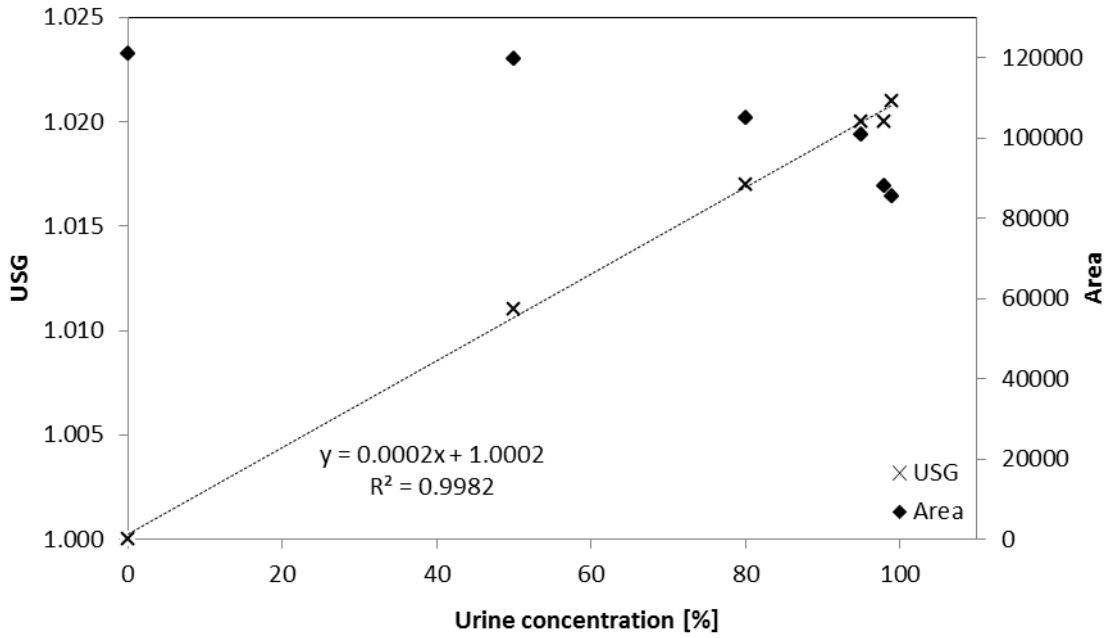


Figure 45 Influence of the USG on the signal suppression caused by the urine matrix in ESMS, 10 µg/L d^9AB (m/z = 188) in differently concentrated urine, 100 % is the undiluted urine, 50 % means urine:water = 1:1, measured with HPLC-ESMS

Table 24 Influence of the USG on the signal suppression of AB in ESMS caused by the urine matrix

Sample	Urine content [%]	USG	%-Area[1]
12204	100	1.021	-[2]
99 + 1[3]	99	1.021	71
49 + 1[3]	98	1.020	73
19 + 1[3]	95	1.020	83
4 + 1[3]	80	1.017	87
1 + 1[3]	50	1.011	99
Ultrapure water	0	1.000	100

[1]Normalised to 10 µg/L d^9AB in ultrapure water, [2]not measured, [3]urine + ultrapure water

The USG and the urine concentration show a quite linear correlation (Table 24 and Figure 45). Concerning the area and furthermore the signal suppression, a non-linear correlation could be observed. The dilution to a urine concentration of 50 % (1 + 1 dilution with ultrapure water) or to an USG of 1.011 led to a quite big signal enhancement. Further dilutions did not show any significant improvements. This means a dilution of urine samples with high USG down to a USG of ~1.01 could improve the measurement of the sample with ESMS a lot.

The total urine matrix in the scan mode showed no big differences caused by dilution (see Figure 46) but at the retention time of AB (~4.5 min) a much lower matrix background could be observed, better visible in Figure 47.

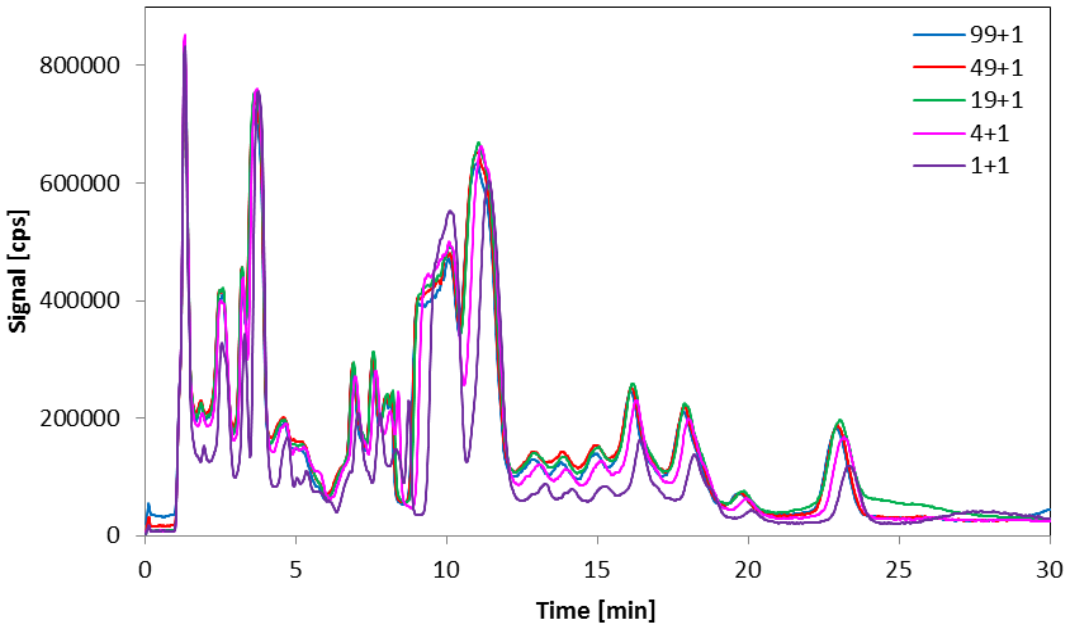


Figure 46 Chromatogram of urine sample 12204 differently diluted (urine + ultrapure water), measured with HPLC-ESMS, scan from m/z = 50-250

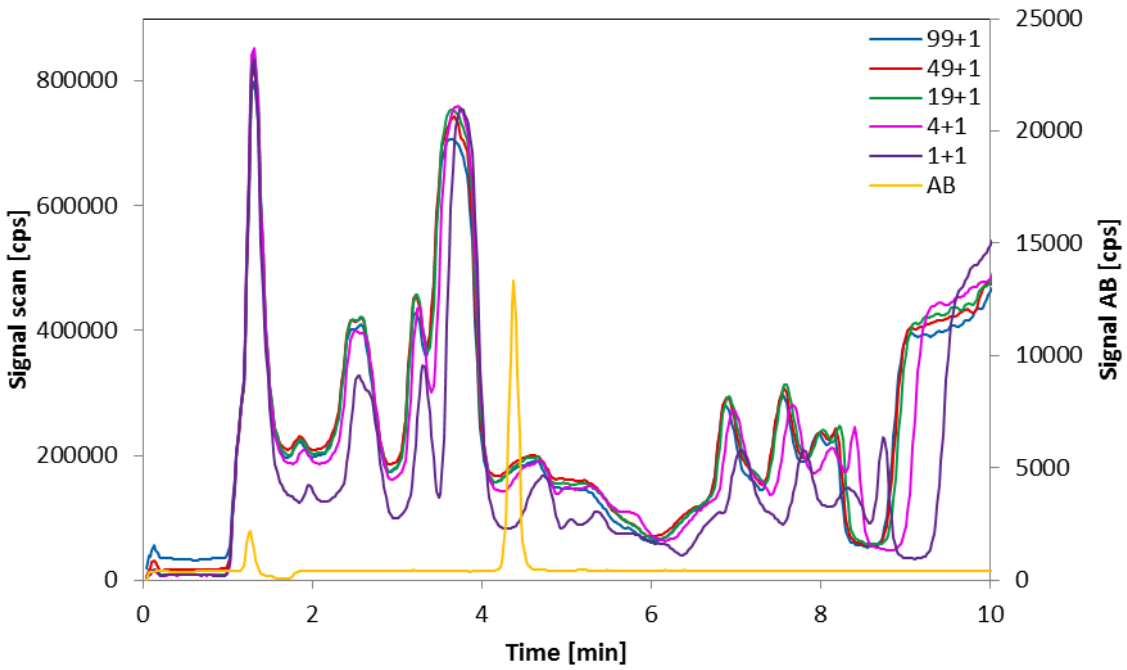


Figure 47 Chromatogram of urine sample 12204 differently diluted (urine + ultrapure water), measured with HPLC-ESMS, scan from m/z = 50-250, the yellow line: AB m/z = 188 (d^9AB) extracted from SIM from ultrapure water spiked with 10 µg/L d^9AB

Having a closer look to the signal of AB, a peak broadening could be observed besides the signal suppression. The broadening caused by the urine matrix is a further problem because the already small peaks can be integrated worse. Different mixtures of urine and ultrapure water were spiked with 10 µg/L d^9AB and measured with HPLC-ESMS. The peaks of d^9AB were integrated and their height and width were compared (see Figure 48). The biggest differences could be observed between water and a urine - water mixture of 1:1. Increasing the urine concentration in the samples up to 80 % led again to a peak broadening and a decrease of the peak height. Between urine concentrations of 80 % to 99 % no big differences could be observed any more.

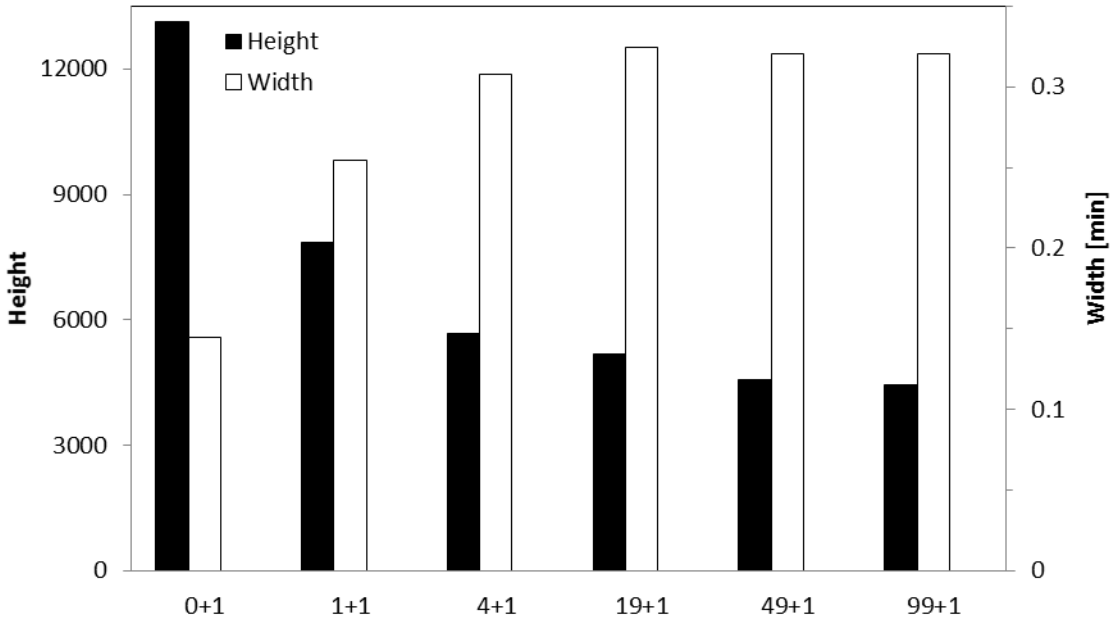


Figure 48 Height and width of the peaks of 10 µg/L d^9AB in urine sample 12204 differently diluted (urine + ultrapure water), measured with HPLC-ESMS, m/z = 188 extracted from SIM and integrated

4.5.2 Different urine matrix compounds and their behaviour in ESMS

Solutions containing different urine matrix compounds and 10 µg/L AB were measured with HPLC-ESMS to investigate their influence on the signal suppression of the urine matrix in our method. To compare the results ultrapure water spiked with 10 µg/L AB was measured as a sample with no signal suppression. The results can be found in Table 25.

Table 25 Signal suppression of different compounds of the urine matrix when measuring AB (10 µg/L) with HPLC-ESMS

Matrix compound	%-Area[1]	Matrix compound	%-Area[1]
NaCl	100	Urea	95
KCl	100	Uric acid	100
$CaCl_2 \cdot 2H_2O$	100	Glycine	98
$MgCl_2 \cdot 6H_2O$	100	Lysine	98
NH_4Cl	100	Glutamine	99
NH_4Cl	99	Histidine	99
$(NH_4)_2HPO_4$	97	Sodium oxalate	99
$(NH_4)_2SO_4$	97		

[1]normalised to 10 µg/L AB in ultrapure water

Urea started eluting quite early (1.2 min), but because of its peak shape (see Figure 49) it influenced the signal of AB. Uric acid (1.3 min), lysine (1.2 min) and glutamine (2.7 min) eluted before AB (4.4 min) and histidine much later. The solutions containing the different

cations did not show any signal suppression, not even NaCl. In the solutions containing NaCl and KCl peaks at 7 min and 10 min could be observed. The peak at 7 min from the NaCl solution was mainly m/z = 91 and a bit m/z = 159. The peak at 10 min from the KCl solution was mainly m/z = 122. The identification of these peaks was not investigated. A measurement of a mixture of these compounds would have been interesting as well, to investigate their interactions. A chromatogram containing the scan from some urine matrix compounds containing 100 µg/L AB is shown in Figure 49.

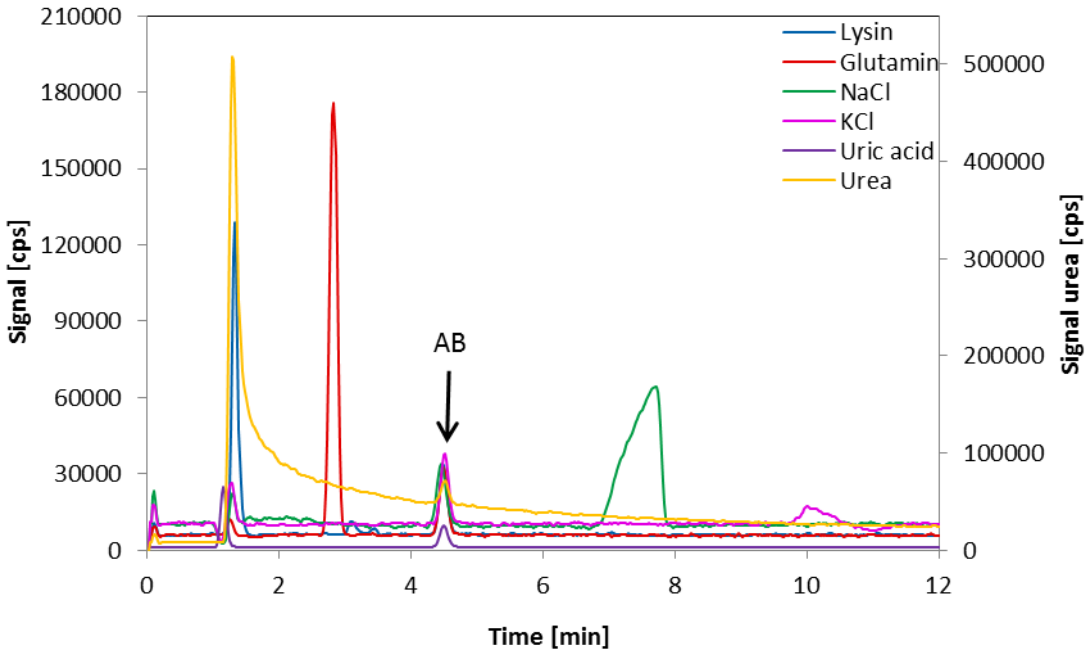


Figure 49 Chromatogram of different urine matrix compounds spiked with 100 µg/L AB (uric acid spiked with 10 µg/L AB), measured with HPLC-ESMS, scan from m/z = 50-250

Unfortunately the urine matrix itself is much more complex than expected. The investigated matrix compounds represent the urine matrix poorly. As seen in Figure 50 many compounds of the urine matrix are not identified. Much more work is necessary to solve the complex composition of human urine.

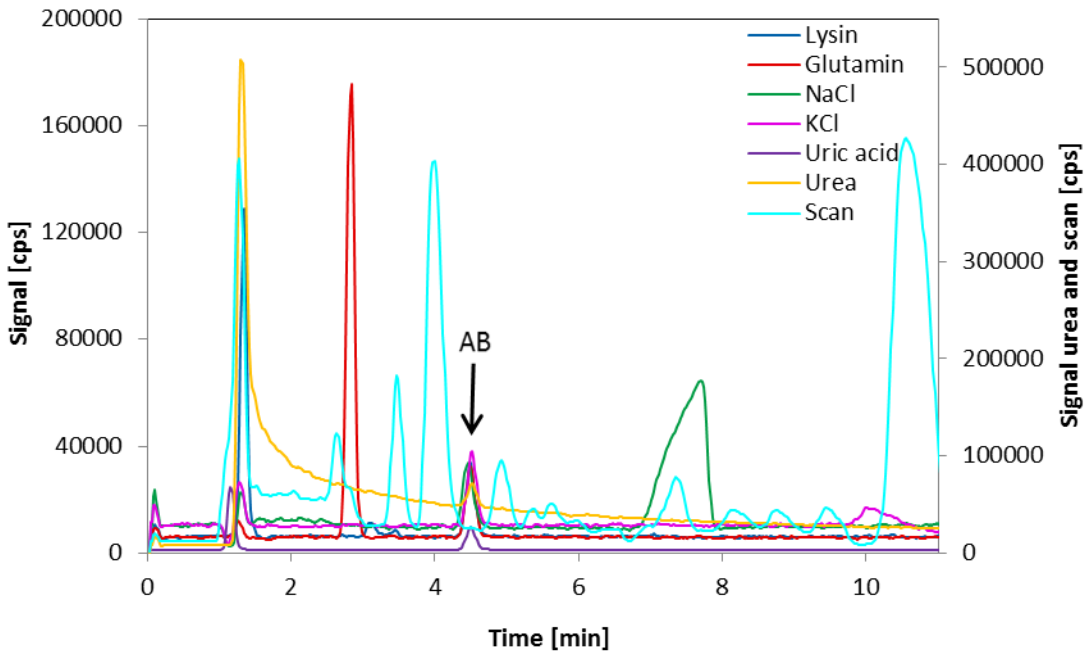


Figure 50 Chromatogram of different urine matrix compounds spiked with 100 µg/L AB (uric acid spiked with 10 µg/L AB) compared with the scan of urine sample 32210 (USG = 1.005) spiked with 10 µg/L AB, measured with HPLC-ESMS, scan from m/z = 50-250

A next step of solving the complex urine matrix is to extract the different m/z building up the matrix. We investigated in the m/z between 58 and 206 and extracted the major ones from the scan of a urine sample to generate a very complex chromatogram, shown in Figure 51. Ultrapure water was measured as well to enable a subtraction of the blank peaks. However, no big peaks were detected in the blank, hence no correction was necessary.

The colour explosion of Figure 51 illustrates the complexity of the urine matrix very well. The big green peak at ~1 min is the urea, already investigated in former experiments. At ~2.5 min two overlying peaks with m/z = 140 and 144 could be observed. The big purple peak at ~4 min has a m/z = 138 and the big pink one at ~11 min a m/z = 114. The ocher peak at ~12 min is a molecule with m/z = 76. The identification of these peaks would have been too much for this master thesis.

To get a better overview we summed up all these chromatograms (from m/z 58-206) and compared them with the scan of the urine matrix (Figure 52).

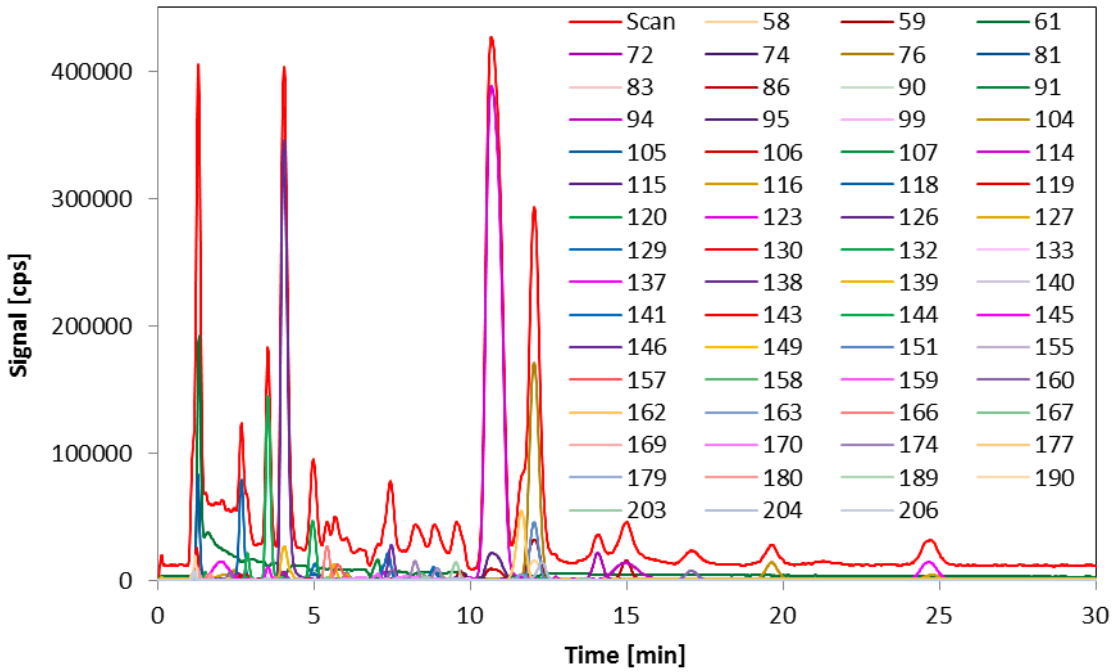


Figure 51 Chromatogram of urine sample 32210, measured with ESMS, the major m/z from the urine matrix were extracted from the scan

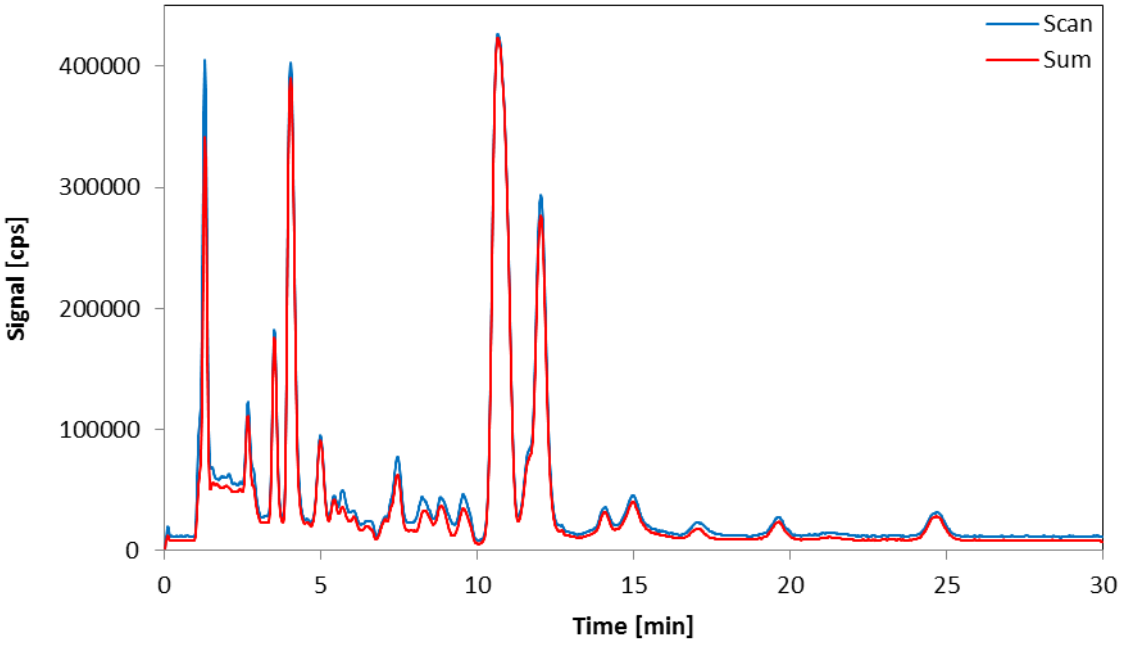


Figure 52 Chromatogram of urine sample 32210 measured with ESMS, red = sum of the matrix compounds between m/z = 58 and 206

The extracted mass to charge ratios (58-206) describe the urine matrix quite well, resulting in a good fit of the two lines in Figure 52. Only small differences in the beginning of the chromatogram could be observed. But these differences are in the error range of the measurement.

From the known matrix compounds the influence of urea was the highest. For this reason a further experiment was performed to investigate if its signal suppression is always the same. Therefore, the urea solution was spiked with different amounts of AB (1-100 µg/L) and compared with ultrapure water spiked with the same amounts of AB.

Figure 53 and Figure 54 show that the signal suppression is not only influenced by the matrix concentration but by the concentration of AB as well. In samples with high amounts of AB the signal is much more suppressed than in samples containing only low amounts of AB. This dependency on the concentration makes quantifications even more challenging.

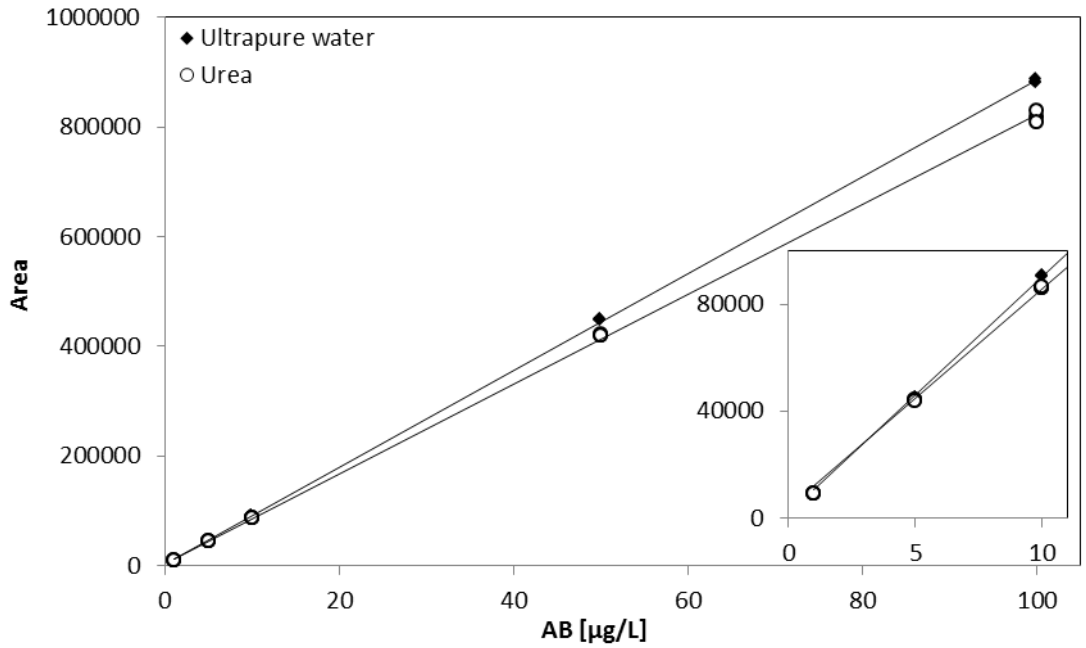


Figure 53 Dependency of the signal suppression in ESMS, on the concentration of AB, caused by urea; Small insert: Same graph as in the big frame, scaled to lower concentrations of AB, measured with HPLC-ESMS

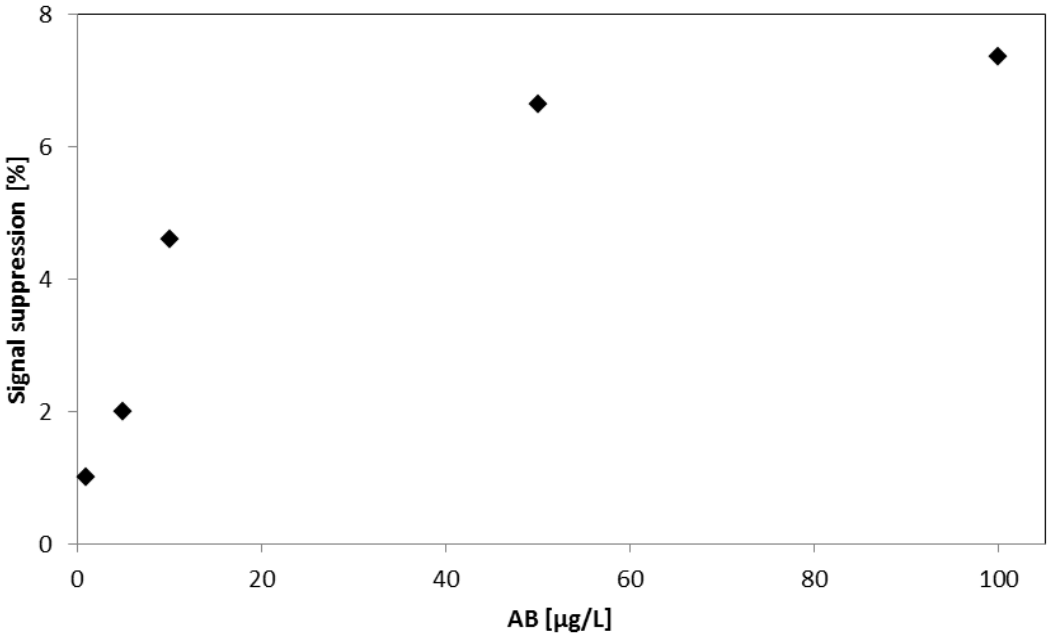


Figure 54 Dependency of the signal suppression in ESMS, on the concentration of AB, caused by urea, measured with HPLC-ESMS

4.6 Results of the Urine Study

4.6.1 Can AB act as a methyl-donor in the one carbon cycle?

To prove if one AB suppresses another, three calibrations ranging from 1-100 µg/L AB were prepared. The first calibration contained only AB, the second only d^9AB and the third both, AB and d^9AB. The integrated areas of the calibrations containing only one form of AB (AB or d^9AB) were compared with the areas in the calibration containing both forms of AB. In the mixed calibration only ~92 % of the areas from the single calibration standards could be found. This means that AB and d^9AB suppressed each other. This could be a problem when focusing on the two partly deuterated ABs (m/z = 182 and 185) because they are supposed to occur in very low amounts.

All urine samples were measured with HPLC-ESMS focusing on m/z = 179, 182, 185 and 188. Starting with the first urine sample after the ingestion of d^9AB high peaks with m/z = 188 could be observed. In some urine samples small peaks with m/z = 179 could be observed as well, probably occurring from the diet. In the chromatograms of m/z = 182 and 185 no peak at the retention time of AB could be observed. This means that no partially deuterated AB could be observed. Exemplary a chromatogram of urine sample 12402 is shown in Figure 55 and Figure 56.

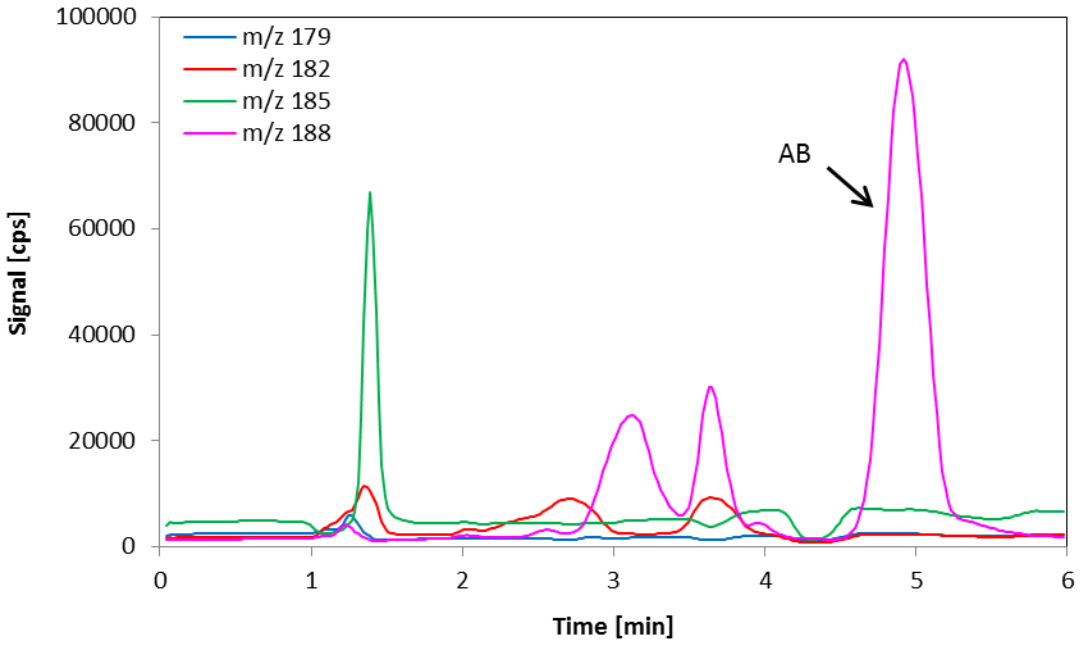


Figure 55 Chromatogram of urine sample 12402 measured with HPLC-ESMS, containing ~270 µg/L d^9AB

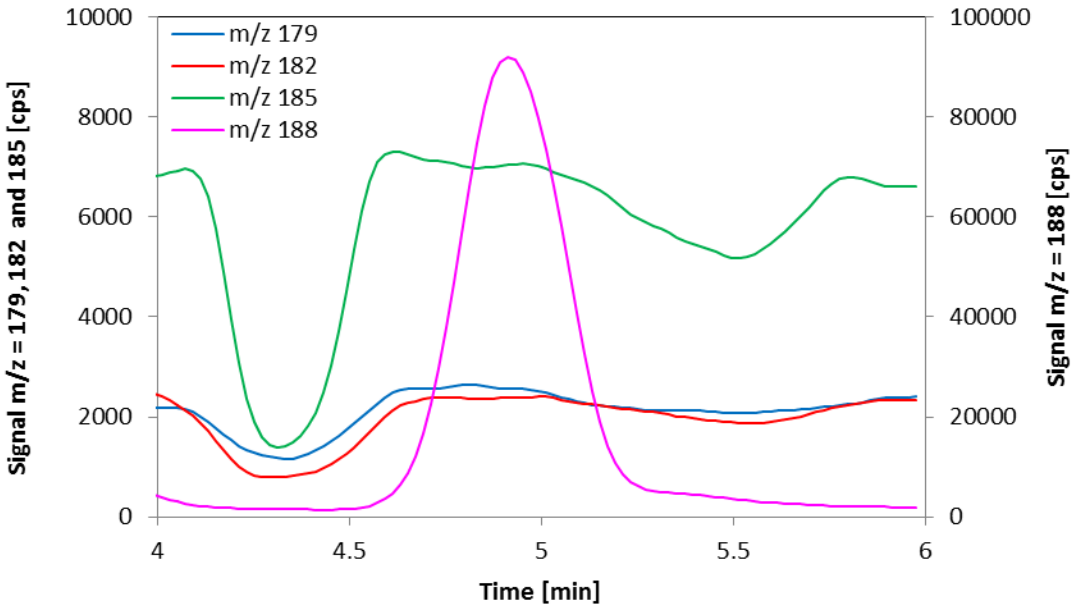


Figure 56 Chromatogram of urine sample 12402 measured with HPLC-ESMS, containing ~270 µg/L d^9AB, zoomed in to the retention time of AB

Dilution of the urine sample would not lead to different results because most of the urine samples from person 3 had USG < 1.010 which is very diluted, and even in these samples no peaks with m/z = 182 and 185 could be observed at the retention time of AB.

To get a better impression of these results the urine samples with the highest concentration of d^9AB from each person, were spiked with AB to determine the amount that is still

observable. We started with a spike resulting in 5 µg/L AB to be sure to observe a peak and ended with a spike resulting in 0.1 µg/L AB in the urine sample. Every concentration was spiked 3 times. The replicates of each spiked concentration did not vary a lot. The results are shown in Table 26 and Figure 57.

Table 26 Results of the spike experiment of the urine samples with the highest amount of d^9AB from all 5 volunteers, spiked with AB, measured with HPLC-ESMS

Urine sample	USG	Observable spiked concentration [µg/L]	% of d^9AB area[1]
12302	1.015	0.1	0.01
22304	1.014	0.5	0.04
32303	1.003	0.1	0.02
42304	1.024	0.5	0.01
52303	1.015	0.5	0.03

[1] % of the area of the lowest observable peak of AB (m/z = 179, compared to the area of the peak of d^9AB (m/z = 188)

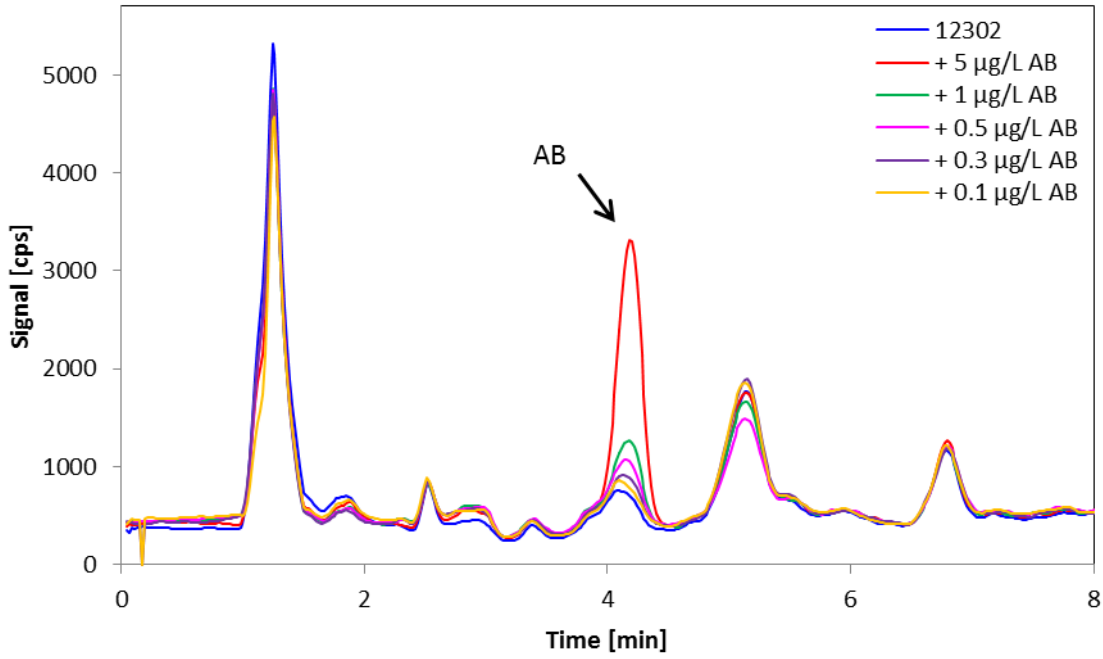


Figure 57 Chromatogram of urine sample 12302 spiked with different concentrations of AB, measured with HPLC-ESMS, m/z = 179

When plotting the integrated areas of the peaks of AB against the spiked concentrations, a linear relationship between the concentrations and the areas is achieved. As an example, for urine sample 12303, shown in Figure 57, the coefficient of determination is $R^2 = 0.9986$.

Summarizing the results of the urine study:

We have shown that more than 99.95 % of the excreted d^9AB was excreted unchanged in the urine samples of all 5 volunteers. There were slight differences occurring from different USG but in none of the urine samples a peak at the retention time of AB with m/z = 182 and 185 (partially deuterated AB) could be observed.

All ABs, regardless of the amount of deuterated methyl-groups, elute at the same time and can suppress the other ABs. Additionally all four ABs suffer from different integration problems. So they cannot be analyzed equally. Standard solutions containing the partially deuterated ABs would enable a determination of the limits of detections of these two compounds.

What are the reasons why we could not observe metabolized AB?

According to Lever and Slow (2010) typical plasma GB levels are between 20 and 75 µmol/L [39]. We ingested 2 mg As in form of d^9AB which are ~27 µmol d^9AB. Calculating with 5-6 L blood in a human body we have ~5 µmol d^9AB per litre blood, hence less than GB. Therefore, one reason why we could not observe any metabolized AB may be the excess of GB. As long as there is enough GB, AB is not metabolized. To proof these assumptions BHMT has to be extracted from the body and only AB should be added to see if AB is metabolised in the absence of GB.

To undergo any reactions, AB has to enter the cells. Randall et al. published 1995, that in *E. coli* AB can be transported in the same way as GB into cells [26], but in 1994 Lever et al. supposed, that the accumulation systems for GB in mammalian systems are more specific than in bacterial ones [111]. In a study, using ^{73}As labeled AB, ingested in mice, rats and rabbits, high concentrations of AB could be found in the kidneys short time after the ingestion but with a fast clearance [31]. Because of this fast clearance an undergoing reaction in the kidneys, for example with BHMT, cannot be the explanation of the second, longer half-life of AB in the human body.

Another reason why AB cannot act as a methyl-donor like GB might be its size. The bond length of As-C is ~1.9 Å, the bond length of N-C is ~1.5 Å. In AB arsenic is bound to 4 C-Atoms and every binding is ~0.4 Å longer than in GB. This leads to a sphere with a volume that is more than twice as big as with N (see Figure 58). Because of this AB might be too big to fit in the active site of the enzyme BHMT.

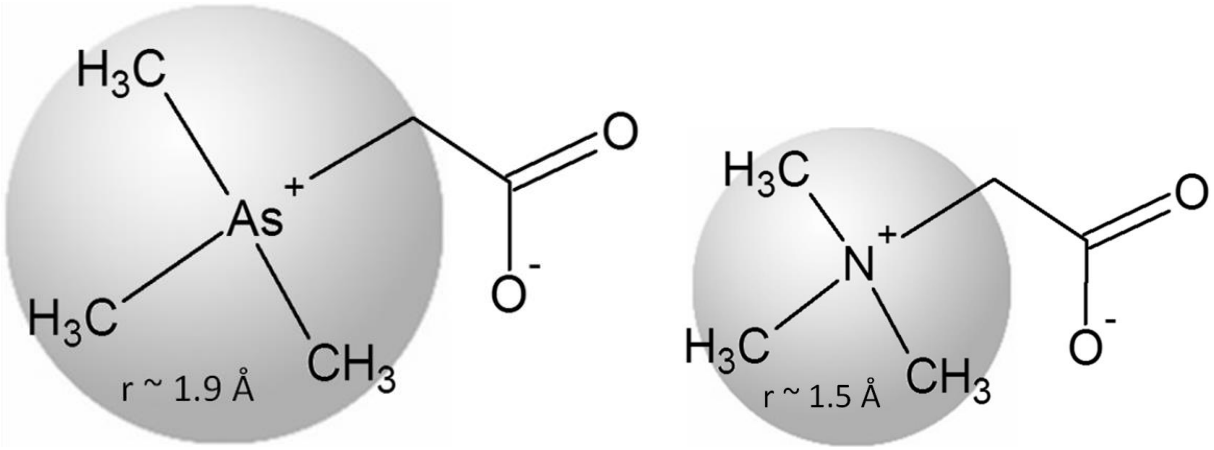


Figure 58 Comparison of the size of AB and GB

4.6.2 Excretion rate and half-life of AB

The total amount of excreted arsenic was plotted against the time after the ingestion, to calculate a half-life and an excretion rate of the ingested arsenic. The fitting was done by Krammer M., University of Technology Graz, Institute of Solid State Physics. Earlier studies from Johnson et al. and Lehmann et al. supposed a first half-life of ~7-11 hours and a second one of ~63-76 hours [29, 30]. Surprisingly one of the 5 volunteers showed a completely different excretion behaviour. The results can be found in Figure 59 to Figure 63 and Table 27.

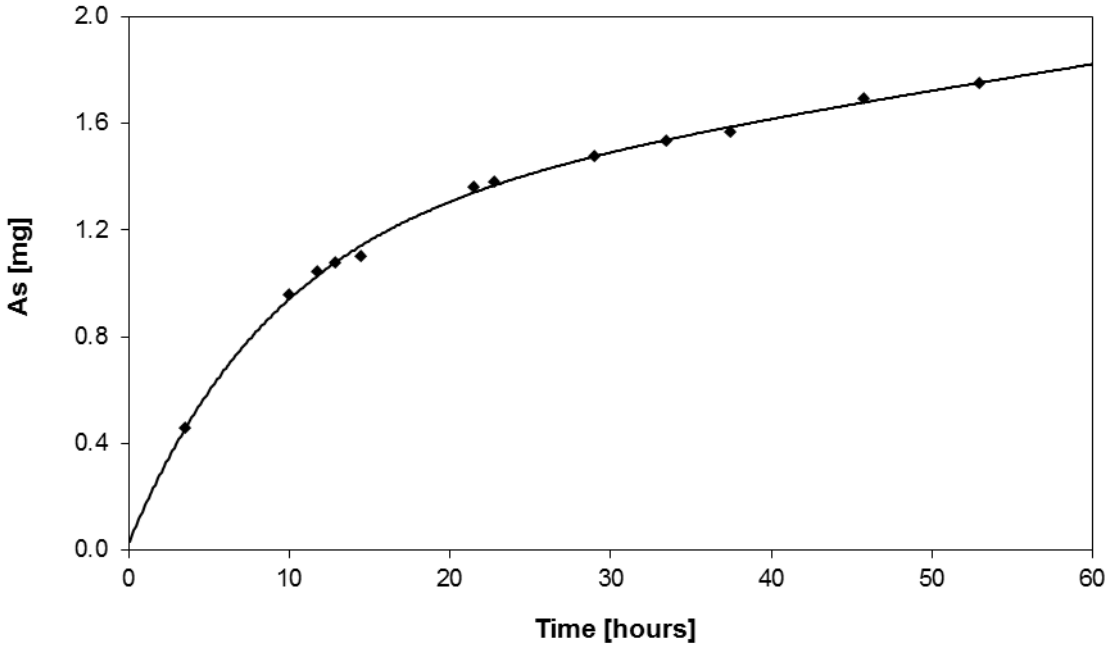


Figure 59 Arsenic excretion profile of person 1 after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

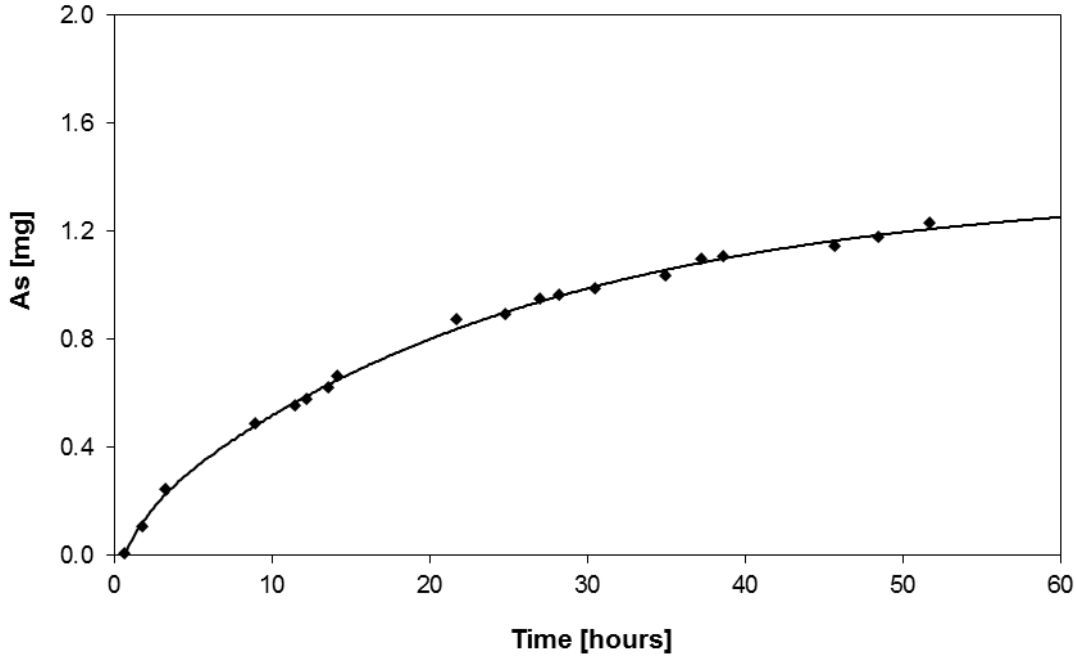


Figure 60 Arsenic excretion profile of person 2 after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

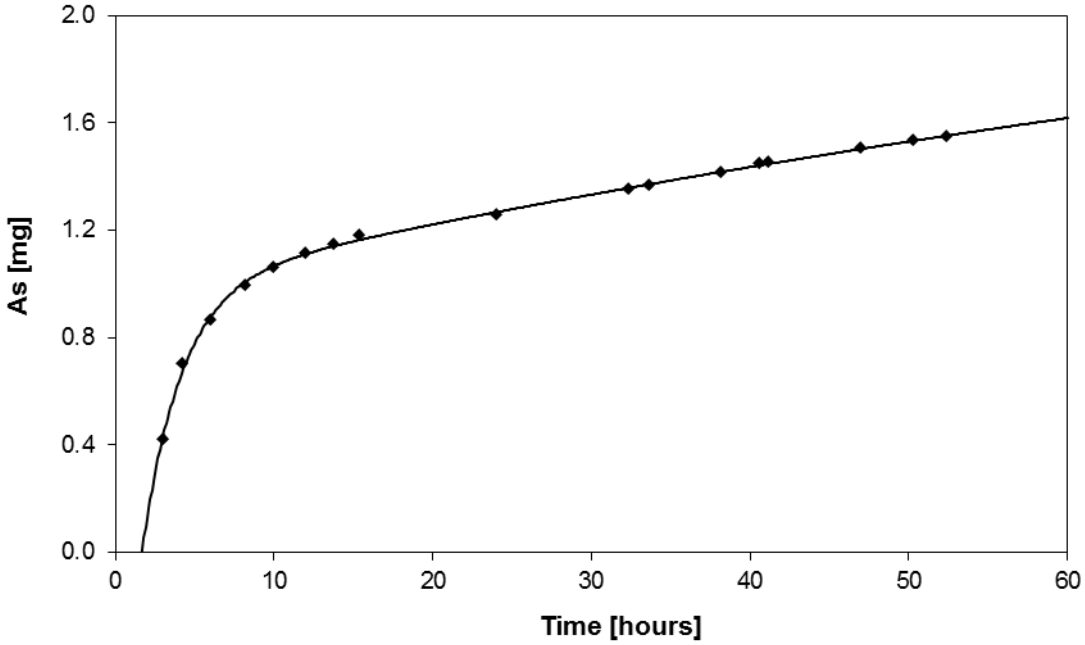


Figure 61 Arsenic excretion profile of person 3 after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

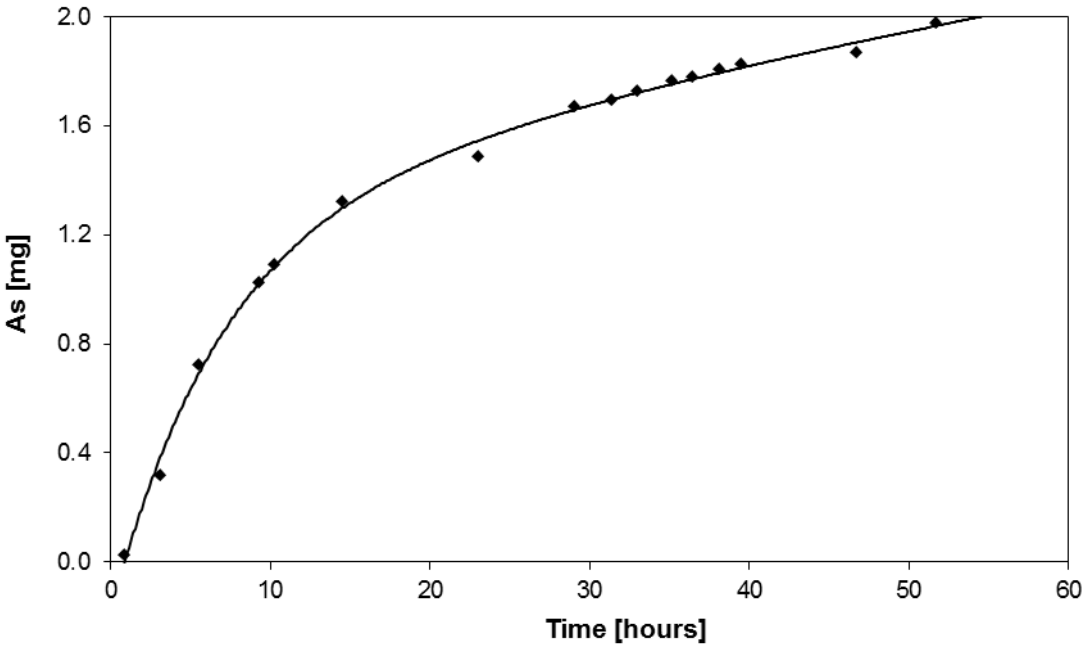


Figure 62 Arsenic excretion profile of person 4 after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

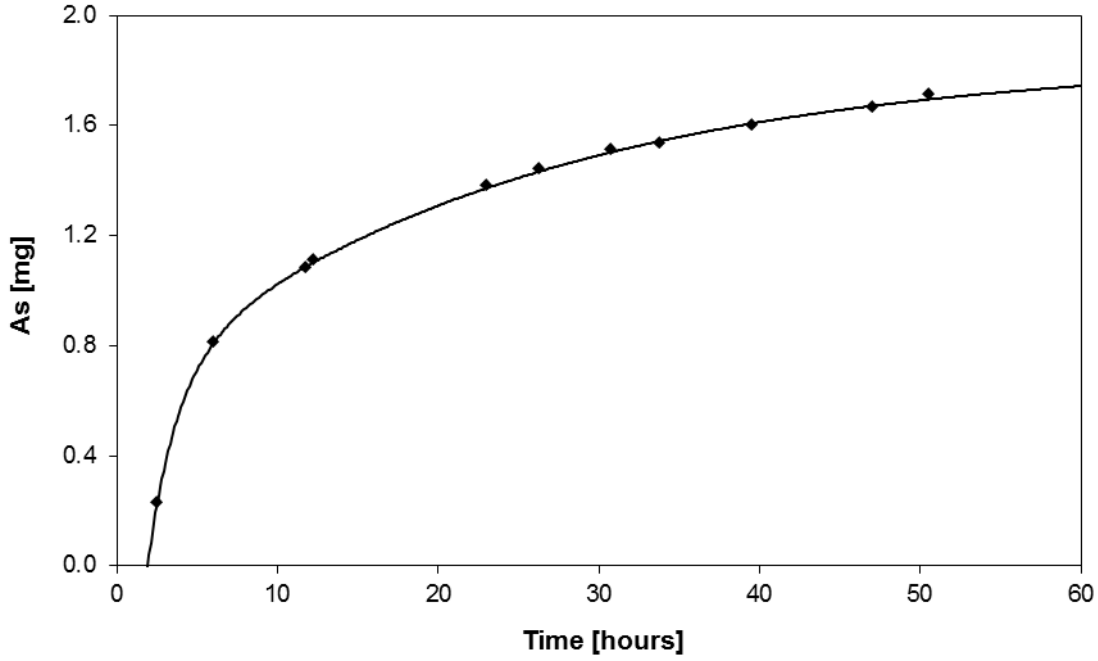


Figure 63 Arsenic excretion profile of person 5 after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

Table 27 Half-lives of AB in the human body

$T_{1/2}$ [hours]	**Person 1**	**Person 2**	**Person 3**	**Person 4**	**Person 5**
Observed[1]	11	31	8	9	9
Calculated[2]	7	15	7	6	8

[1] the time after which 1 mg (the half of the ingested 2 mg) of arsenic was excreted with the urine; [2] half-life calculated with the function fitted through the measured amounts of As

In Table 27 two half-lives are mentioned. The first one, the observed half-life, is the time, in which 1 mg of AB was excreted. The calculated one is calculated from the function, fitted into the measured points. At a closer look none of the curves shown in Figure 59 to Figure 63 start with 0 mg at time 0 hours and most of them do not converge to the ingested 2 mg for long times. Therefore, the calculated half-lives differ a bit from the observed ones, especially for person 2. The urine samples were only collected for 2.5 days and so the collection was not long enough, to calculate a reliable second half-life.

Comparing the half-lives from the 5 volunteers with the literature (~7-11 hours [29, 30]), they agree well except person 2 who showed a higher half-life because she excreted AB slower than the other volunteers.

Excretion rates for all 5 volunteers were calculated and are summarized in Table 28. To get a better impression different time periods were used.

Table 28 Excretion rates of arsenic in different time periods, after the ingestion of 2 mg As in form of d^9AB, measured with ICPMS

Time	**0 h - 5 h** [µg/h]	**0 h -10 h** [µg/h]	**0 h - 15 h** [µg/h]	**15 h - 50 h** [µg/h]	**30 h - 50 h** [µg/h]
Person 1	120	94	77	16	12
Person 2	64	52	45	15	10
Person 3	156	107	77	11	10
Person 4	127	107	88	18	14
Person 5	143	102	79	14	10

As seen in Table 28 the excretions rates are really high at the beginning and get lower after a period of time. For all 5 volunteers the first excretion rate (0-5 hours after the ingestion) was the highest one.

Comparing the excretion rates between the 5 volunteers; person 2 shows a much lower excretion rate of AB than the other 4 volunteers, at least at the beginning. In the time periods from 15-50 h and 30-50 h the excretion rates for all 5 volunteers were quite similar. The excretion rate between 0-5 h of person 3 is the highest noticed one, and when looking at Figure 59 to Figure 63 this result seems to be reliable.

5 Conclusions

SPE is a powerful clean-up tool when working with complex matrices. The developed method, using a LiChrolut® SCX cartridge, conditioned with formic acid, showed quite good results. For a perfect urine clean-up, separating AB from the matrix, more investigations concerning the eluting step have to be done.

In cationic arsenic speciation analysis ammonium formate buffers have shown comparable results to the commonly used pyridine buffer, especially when focusing on AB. Furthermore they showed good performance in HPLC-ESMS. The addition of 65 % of MeOH to a 20 mM ammonium formate buffer (pH 2.5) improved the direct measurement of AB in the urine matrix with HPLC-ESMS.

The normalisation of urine samples by their USG is important to make results comparable. Unfortunately at low USG the correlation between the urine concentration, respectively the dilution of the urine, and the USG is not linear any more. This means, when working with highly diluted urine, this normalisation technique is error-prone. Because no better normalisation technique is known, all urine samples were normalised by their USG.

Concerning the total amount of 28 elements, differences between the 5 volunteers could be observed. All 5 volunteers had the same major elements in their urine but have shown big differences in the overall excreted amounts. Of course this depends on the ingested foods and drinks. Differences between the correlations of some elements in the urine have been observed as well. Some elements showed correlations in all urine samples and others only in urine samples of individual volunteers. Even negative correlations could be observed.

Comparison of cation-exchange and anion-exchange chromatography did not show any big differences in the arsenic speciation analysis of the urine samples and no unknown arsenic species could be observed. Comparison of the speciation analysis and the analysis of the total amount of arsenic with ICPMS showed no big differences either.

Urine consists of many different compounds and many of them can lead to signal suppression in ESMS. Experiments have shown that the major urine compounds cause only a small part of the signals observed in a scan of a urine sample. Further experiments showed that a lot of different compounds can be detected with ESMS. Signal suppression correlated well with the water content of the urine and the USG. In quite concentrated urine (containing 10 µg/L d9AB) up to 30 % of the signal was suppressed. Therefore, dilution would help against signal suppression, if it is possible. Unfortunately different concentrations of AB get suppressed differently. The higher the concentration of AB has been in the urine, the more the signal was suppressed by the matrix. Experiments have shown that m/z = 188 and

m/z = 179 suffer from different matrix effects. Because of this, standard substances from the partially deuterated ABs (m/z = 182 and 185) would support our results a lot to ensure that they do not suffer from big matrix effects. With our method we could detect 5 µg/L d^9AB and 0.5 µg/L AB in highly concentrated urine directly with HPLC-ESMS.

The results of the urine study have shown that less than 0.05 % of the excreted d^9AB were metabolised. The excretion behaviour of AB was comparable with literature, at least for 4 of the 5 volunteers. Person 2 showed a really small excretion rate and should repeat the study to prove this result.

6 Remaining Questions and Outlook

The urine matrix consists of many different compounds and most of them are unknown. Further investigations on the identification of the urine matrix, focusing on the signal suppression in ESMS would be very interesting and could help improving urine analysis.

Another urine study with the volunteer with the low excretion rate of AB would prove the unexpected results. Moreover a longer urine study with more volunteers would be interesting. Are there more people that excrete AB with a half-life >10 h? By collecting the samples over a longer period of time, a better fitting would be possible and the second half-life could be calculated. Furthermore a controlled diet for all volunteers, participating at the study, would be interesting, especially when focusing on the elemental composition if their urine.

Synthesising standard compounds of the partially deuterated ABs (m/z = 182 and 185) would enable an investigation of the influence of the urine matrix on these two compounds and a determination of an LOD for each AB.

To assure that AB cannot act as a methyl donor in the one carbon cycle an in vitro experiment should be performed. There AB could be added without any disturbing substances or competitions between AB and GB.

7 Abbreviations

AB	Arsenobetaine bromide
AC	Arsenocholine
APCI	Atmospheric pressure chemical ionization
APPI	Atmospheric pressure photoionization
As(III)	Arsenite
As(V)	Arsenate
BHMT	Betaine-homocysteine-methyltransferase
BMI	Body mass index
d^9AB	Deuterated arsenobetaine bromide
DMA	Dimethylarsinate
DMA(III)	Dimethylarsinite
EI	Electron ionization
ES	Electrospray
ESI	Electro spray ionization
ESMS	Electro spray mass spectrometry
FD-ESI	Fused-droplet electrospray ionization
GB	Glycine betaine, betaine, trimethylglycine
HPLC	High performance liquid chromatography
ICPMS	Inductively coupled plasma mass spectrometry
m/z	Mass to charge ratio
MA	Methylarsonate
MA(III)	Methylarsonite
MALDI	Matrix assisted laser desorption/ionization
MeOH	Methanol
MTR	Methyltetrahydrofolate-homocysteine-methyltransferase
S/N	Signal to noise ratio
SAM	S-adenosylmethionine
SAX	Strong anion-exchange
SCX	Strong cation-exchange
SPE	Solid phase extraction
TETRA	Tetrametylarsonium ion
TFA	Trifluoroacetic acid
THF	Tetrahydrofolate
TMAO	Trimethylarsine oxide
UER	Urinary excretion rate
USG	Urine specific gravity
v/v	Volume/volume (percent by volume)

Code of the urine samples: abbcc

a...number of the person: 1-5

b…the date of the sampling: 22-25 because the samples were collected between the 22th and 25th of July 2014

c…the number of the sample on a day: 01 is the first sample on a day

Example:42406 is the 6th urine sample from person 4 on the 24th of July

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9 Appendix

Information about the urine samples

Table 29 Information of the urine samples, the sample name consist of the person (1-5), the date (22-25) and the sample number (01-10), AB was ingested on the 23th of July at 8:00 o'clock

Sample	Time	Amount [mL]	USG	Sample	Time	Amount [mL]	USG
12201	05:30	600	1.014	32303	11:00	1000	1.003
12202	08:45	520	1.008	32304	12:15	720	1.003
12203	13:00	510	1.011	32305	14:00	710	1.003
12204	21:10	480	1.021	32306	16:10	810	1.005
12301	05:30	650	1.019	32307	17:55	300	1.006
12302	11:50	480	1.015	32308	20:02	500	1.004
12303	18:00	640	1.012	32309	21:48	300	1.006
12304	19:45	800	1.004	32310	23:22	390	1.005
12305	20:50	650	1.002	32401	07:53	400	1.012
12306	22:30	400	1.004	32402	16:20	700	1.009
12401	05:30	800	1.014	32403	17:40	180	1.007
12402	06:45	110	1.016	32404	22:10	860	1.006
12403	13:00	510	1.015	32501	00:35	990	1.004
12404	17:30	230	1.024	32502	01:12	100	1.007
12405	21:30	200	1.025	32503	07:00	810	1.008
12501	05:45	480	1.028	32504	10:20	990	1.004
12502	13:00	300	1.027	32505	12:25	260	1.007
22201	05:30	300	1.016	42202	13:20	120	1.026
22202	06:40	30	1.020	42203	17:05	60	1.016
22203	08:40	100	1.020	42204	20:05	110	1.024
22204	11:00	300	1.006	42205	21:05	190	1.007
22205	14:00	200	1.011	42206	23:15	120	1.014
22206	19:00	150	1.020	42301	06:55	100	1.024
22207	19:40	200	1.005	42302	08:50	260	1.005
22208	21:35	150	1.009	42303	11:05	270	1.007
22301	05:45	300	1.013	42304	13:30	100	1.017
22302	08:40	400	1.003	42305	17:15	110	1.013
22303	09:50	200	1.007	42306	18:15	20	1.019
22304	11:15	100	1.014	42307	23:30	150	1.018
22305	17:00	200	1.020	42401	07:00	100	1.025
22306	19:30	100	1.022	42402	13:50	160	1.022
22307	20:15	250	1.004	42403	15:25	230	1.005
22308	21:35	300	1.005	42404	17:00	120	1.010
22309	22:10	400	1.003	42405	19:10	70	1.020
22401	05:45	400	1.017	42406	20:25	200	1.006
22402	08:50	50	1.042	42407	22:10	330	1.006

Sample	Time	Amount [mL]	USG	Sample	Time	Amount [mL]	USG
22403	11:00	200	1.013	42408	23:30	100	1.012
22404	12:15	150	1.006	42501	06:45	125	1.021
22405	14:30	200	1.007	42502	11:45	200	1.023
22406	19:00	400	1.007	52201	07:30	300	1.020
22407	21:15	300	1.013	52202	11:30	280	1.02
22408	22:40	250	1.005	52203	17:00	300	1.024
22501	05:45	300	1.014	52204	23:30	250	1.020
22502	08:30	150	1.020	52205	01:00	350	1.005
22503	11:45	200	1.030	52301	07:30	250	1.020
32201	06:00	300	1.009	52302	10:30	300	1.008
32202	09:38	300	1.006	52303	14:00	300	1.015
32203	11:00	650	1.002	52304	19:45	650	1.009
32204	12:58	1000	1.002	52305	20:15	500	1.002
32205	13:35	650	1.002	52401	07:00	550	1.017
32206	15:45	600	1.003	52402	10:15	220	1.018
32207	16:55	660	1.003	52403	14:45	250	1.020
32208	19:50	870	1.004	52404	17:46	250	1.008
32209	21:54	650	1.004	52405	23:30	300	1.021
32210	22:33	110	1.005	52501	07:00	300	1.018
32301	01:20	500	1.006	52502	10:30	250	1.020
32302	06:30	310	1.011				

Table 30 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 1/7 samples 12201 - 12502

Sample		12201	12202	12203	12204	12301	12302	12303	12304	12305	12306	12401	12402	12403	12404	12405	12501	12502
USG		1.014	1.008	1.011	1.021	1.019	1.015	1.012	1.004	1.002	1.004	1.014	1.016	1.015	1.024	1.025	1.028	1.027
Li	**[µg/L]**	380	23	31	59	290	30	54	28	18	26	77	72	61	130	96	110	92
B	**[µg/L]**	810	300	440	790	1500	880	610	170	120	190	620	607	619	1070	760	1400	1500
Na	**[mg/L]**	2600	1300	1100	2300	2800	1300	940	580	350	430	2200	1966	1220	2153	2200	3400	1800
Mg	**[mg/L]**	70	26	45	79	69	68	58	22	19	29	76	79	80	123	160	180	160
P	**[mg/L]**	720	150	350	1100	1100	440	690	220	120	210	760	572	439	1515	2100	2800	1800
S	**[mg/L]**	570	280	330	700	640	480	390	120	75	130	620	724	600	1176	1400	1300	1100
K	**[mg/L]**	920	1200	1800	3100	2300	2600	1800	400	140	210	1500	3156	2968	4931	4300	3400	4900
Ca	**[mg/L]**	83	40	59	94	62	77	62	24	20	27	91	77	95	143	140	160	160
V	**[µg/L]**	1.3	0.9	1.0	1.1	1.0	1.1	0.6	0.4	0.2	0.2	0.7	1.1	0.9	1.4	0.9	0.8	1.2
Cr	**[µg/L]**	0.5	0.9	0.6	0.5	1.1	1.7	0.5	1.0	0.5	1.2	1.5	0.9	0.5	1.4	0.6	0.8	0.9
Mn	**[µg/L]**	1.0	0.7	0.4	0.5	0.6	0.6	0.5	0.5	0.6	0.4	0.6	0.3	0.4	1.4	0.6	0.7	0.6
Fe	**[µg/L]**	100	19	15	19	95	24	15	18	12	13	20	21	18	161	21	23	27
Co	**[µg/L]**	0.3	0.2	0.2	0.4	0.5	0.3	0.2	<0.2	<0.2	<0.2	0.2	0.2	0.2	0.5	0.6	0.4	0.4
Ni	**[µg/L]**	<1	<1	1.6	2.2	1.9	1.4	1.6	<1	1.8	<1	3.9	5.2	3.2	5.1	5.6	4.7	6.0
Cu	**[µg/L]**	13	9.4	13	17	13	14	11	7.2	7.1	7.5	13	14	12	15	18	16	19
Zn	**[µg/L]**	220	140	210	460	360	370	270	76	48	91	300	392	361	584	1100	720	760
As	**[µg/L]**	20	15	16	21	21	970	800	120	68	92	340	272	210	284	230	280	230
Se	**[µg/L]**	25	17	24	43	29	30	22	10	6.6	9.2	23	29	27	51	61	44	58
Rb	**[mg/L]**	0.9	1.0	1.9	3.5	2.4	2.6	1.9	0.4	0.1	0.2	1.4	3.0	3.0	4.8	4.6	3.4	4.8
Sr	**[µg/L]**	230	110	160	250	160	200	170	80	66	90	260	235	266	392	400	490	450
Mo	**[µg/L]**	15	8.4	15	42	150	130	40	7.6	4.7	11	46	43	23	41	31	77	94
Cd	**[µg/L]**	0.3	0.2	0.3	0.6	0.5	0.8	0.4	0.1	0.1	0.1	0.3	0.5	0.5	0.7	1.1	0.9	1.1
Sb	**[µg/L]**	0.1	<0.1	0.1	0.1	0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	0.1	0.1	0.1	0.1
Te	**[µg/L]**	0.3	<0.2	<0.2	0.4	0.2	<0.2	0.4	0.2	<0.2	<0.2	0.5	0.2	0.3	<0.2	0.4	0.3	0.2
Ba	**[µg/L]**	4.2	3.3	3.2	3.8	3.2	3.4	4.3	4.3	3.8	4.1	5.2	5.8	4.4	5.3	9.6	5.6	4.8
Hg	**[µg/L]**	1.3	1.3	1.3	1.4	0.9	1.7	0.9	0.8	0.4	0.8	1.1	1.3	0.9	1.2	1.5	2.0	1.7
Pb	**[µg/L]**	1.3	1.2	1.3	1.8	1.6	1.5	1.4	1.1	1.4	1.2	1.7	1.7	1.5	2.3	2.1	2.0	2.2
Bi	**[µg/L]**	0.1	<0.1	<0.1	0.4	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
U	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1

Table 31 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 2/7 samples 22201 - 22308

Sample		22201	22202	22203	22204	22205	22206	22207	22208	22301	22302	22303	22304	22305	22306	22307	22308
USG		1.016	1.020	1.020	1.006	1.011	1.020	1.005	1.009	1.013	1.003	1.007	1.014	1.020	1.022	1.004	1.005
Li	**[µg/L]**	370	23	21	10	22	47	11	16	290	3.8	11	24	50	48	6.6	9.5
B	**[µg/L]**	1200	740	720	240	410	770	250	500	1200	160	330	740	1300	1300	200	220
Na	**[mg/L]**	1900	2500	3300	620	1200	1800	790	2600	2100	150	890	1400	1500	2700	370	830
Mg	**[mg/L]**	74	160	180	43	85	130	43	76	65	22	54	100	110	92	26	44
P	**[mg/L]**	1100	590	73	24	230	940	140	250	910	9.4	6.0	77	910	1900	190	140
S	**[mg/L]**	650	720	700	180	350	910	210	350	580	110	170	450	800	1000	150	180
K	**[mg/L]**	1200	1800	2400	690	1600	2600	230	460	1600	180	670	3000	3500	2500	210	210
Ca	**[mg/L]**	40	120	220	51	88	93	57	110	31	36	110	150	88	53	25	70
V	**[µg/L]**	0.7	1.2	1.9	0.6	0.9	1.0	0.3	0.8	0.8	0.2	0.5	1.2	0.9	0.8	0.1	0.3
Cr	**[µg/L]**	0.8	0.7	1.0	0.7	1.3	0.5	0.7	0.8	0.8	0.6	<0.5	0.6	1.0	0.6	<0.5	<0.5
Mn	**[µg/L]**	0.7	0.8	0.6	3.9	2.4	1.8	0.3	1.1	0.8	0.8	1.2	0.4	1.5	0.8	0.2	0.6
Fe	**[µg/L]**	120	21	22	56	77	40	15	25	98	20	27	8.2	21	14	16	8.2
Co	**[µg/L]**	0.4	0.4	0.5	0.2	0.4	0.7	0.2	0.2	0.5	<0.2	<0.2	0.3	0.8	0.6	<0.2	<0.2
Ni	**[µg/L]**	3.8	2.8	2.2	<1	2.9	4.0	1.3	1.5	3.7	4.3	3.1	2.2	11	3.8	3.2	<1
Cu	**[µg/L]**	15	19	15	8.8	11	15	6.2	7.4	12	6.1	8.9	7.2	33	13	4.7	3.7
Zn	**[µg/L]**	290	590	350	110	150	160	57	72	180	44	47	47	240	210	56	66
As	**[µg/L]**	12	15	13	5.4	7.5	12	4.4	6.8	8.7	27	500	1400	1200	700	93	150
Se	**[µg/L]**	33	39	38	12	20	31	8.3	12	21	6.4	11	21	33	40	8.4	11
Rb	**[mg/L]**	1.3	1.8	1.6	0.5	1.0	2.0	0.2	0.3	1.3	0.1	0.5	1.9	2.8	2.2	0.2	0.2
Sr	**[µg/L]**	76	220	310	84	150	180	82	140	71	53	140	210	190	120	46	96
Mo	**[µg/L]**	94	100	66	22	48	130	20	17	26	5.3	9.4	22	79	86	15	17
Cd	**[µg/L]**	0.3	0.6	0.3	0.2	0.3	0.3	0.1	0.1	0.1	0.1	0.1	0.2	0.6	0.3	0.1	0.1
Sb	**[µg/L]**	0.1	0.1	0.1	<0.1	0.2	0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	0.2	0.1	0.1	<0.1
Te	**[µg/L]**	<0.2	<0.2	0.2	0.3	<0.2	0.4	<0.2	<0.2	<0.2	0.2	<0.2	<0.2	0.3	0.2	<0.2	<0.2
Ba	**[µg/L]**	4.4	13	11	5.5	15	8.0	5.0	6.9	4.6	3.9	5.9	6.9	7.8	5.4	3.2	4.4
Hg	**[µg/L]**	2.9	3.2	1.8	0.7	1.3	1.6	0.6	0.4	1.3	0.8	0.9	0.9	1.2	1.5	0.9	0.7
Pb	**[µg/L]**	2.1	2.4	2.2	1.8	2.5	2.7	1.2	1.6	1.9	1.2	1.6	1.8	6.3	2.4	1.4	1.6
Bi	**[µg/L]**	<0.1	<0.1	<0.1	0.1	0.1	0.5	<0.1	<0.1	0.2	<0.1	<0.1	<0.1	1.0	0.1	0.2	<0.1
U	**[µg/L]**	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 32 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 3/7 samples 22309 - 32204

Sample		22309	22401	22402	22403	22404	22405	22406	22407	22408	22501	22502	22503	32201	32202	32203	32204
USG		1.003	1.017	1.042	1.013	1.006	1.007	1.007	1.013	1.005	1.014	1.020	1.030	1.009	1.006	1.002	1.002
Li	**[µg/L]**	5.3	28	25	18	10	18	15	27	7.7	170	29	34	360	8.7	3.9	3.2
B	**[µg/L]**	120	730	720	490	210	3330	340	560	170	920	710	800	1100	320	130	120
Na	**[mg/L]**	510	2800	3100	1700	570	610	980	2100	740	1900	2900	2400	1900	340	260	270
Mg	**[mg/L]**	28	70	180	90	27	74	56	56	11	61	140	180	38	20	8.3	6.4
P	**[mg/L]**	56	1300	200	64	120	160	190	570	210	990	420	350	570	91	55	36
S	**[mg/L]**	89	800	680	210	200	290	270	560	200	610	720	850	300	220	100	69
K	**[mg/L]**	72	1500	1500	2500	1200	620	490	990	390	920	2000	2600	760	970	480	470
Ca	**[mg/L]**	50	100	260	150	32	95	87	71	8.9	31	130	230	22	14	13	13
V	**[µg/L]**	0.2	0.8	1.3	1.1	0.5	0.4	0.5	0.7	0.2	0.5	1.4	1.3	0.9	0.3	0.3	0.2
Cr	**[µg/L]**	0.5	1.6	1.0	1.2	0.6	0.6	1.3	0.7	0.7	1.7	2.7	0.8	0.7	0.5	1.2	<0.5
Mn	**[µg/L]**	0.4	0.4	1.0	0.5	0.3	0.5	0.3	0.8	0.7	2.3	2.0	0.9	0.3	0.2	0.7	<0.1
Fe	**[µg/L]**	32	17	37	16	16	12	11	69	10	75	64	130	120	8.5	43	3.2
Co	**[µg/L]**	<0.2	0.5	0.5	0.3	0.2	0.2	0.2	0.4	<0.2	0.3	0.5	0.5	0.5	0.2	<0.2	<0.2
Ni	**[µg/L]**	2.0	1.9	8.6	4.3	7.5	1.6	1.4	2.6	1.1	6.0	3.4	3.4	2.3	1.5	1.3	<1
Cu	**[µg/L]**	4.5	12	42	7.3	4.6	5.9	3.7	6.9	3.0	13	16	14	7.5	6.8	5.8	3.3
Zn	**[µg/L]**	47	180	330	88	38	81	54	160	20	200	210	220	66	64	27	8.1
As	**[µg/L]**	110	520	500	280	100	140	110	210	52	140	220	280	6.5	4.5	3.0	3.2
Se	**[µg/L]**	6.6	34	33	23	10	15	11	21	7.3	26	41	44	16	14	5.7	5.3
Rb	**[mg/L]**	0.1	1.5	1.2	1.5	0.7	0.5	0.4	0.8	0.3	1.0	1.9	1.9	0.7	0.9	0.3	0.3
Sr	**[µg/L]**	64	150	370	200	56	140	110	130	19	68	220	340	75	47	37	31
Mo	**[µg/L]**	9.2	82	60	32	12	29	22	37	7.4	34	42	42	21	9.6	5.1	3.4
Cd	**[µg/L]**	0.1	0.3	0.8	0.2	0.1	0.1	0.1	0.2	<0.1	0.2	0.4	0.3	0.1	0.1	<0.1	<0.1
Sb	**[µg/L]**	0.1	0.1	0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.3	0.3	0.1	0.1	0.1	0.2	<0.1
Te	**[µg/L]**	<0.2	0.2	0.4	<0.2	<0.2	<0.2	0.2	<0.2	0.3	0.2	<0.2	0.3	<0.2	0.4	0.3	0.2
Ba	**[µg/L]**	4.1	5.4	14	6.4	2.9	5.6	4.2	4.9	2.1	5.5	10	10	2.0	1.9	6.2	1.3
Hg	**[µg/L]**	0.6	2.9	1.5	0.9	0.7	0.7	0.6	0.8	0.6	2.6	2.5	1.7	0.9	1.0	0.5	0.3
Pb	**[µg/L]**	1.7	2.5	4.8	2.1	1.6	2.0	1.6	2.4	1.5	3.1	3.4	2.8	1.6	1.5	3.9	1.3
Bi	**[µg/L]**	<0.1	<0.1	0.6	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	0.1	<0.1	<0.1	<0.1	0.1	<0.1
U	**[µg/L]**	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 33 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 4/7 samples 32205 - 32310

Sample		32205	32206	32207	32208	32209	32210	32301	32302	32303	32304	32305	32306	32307	32308	32309	32310
USG		1.002	1.003	1.003	1.004	1.004	1.005	1.006	1.011	1.003	1.003	1.003	1.005	1.006	1.004	1.006	1.005
Li	**[µg/L]**	4.0	3.7	2.9	4.6	7.0	16	21	320	8.0	7.6	6.2	6.7	11	11	16	10
B	**[µg/L]**	160	160	140	230	250	290	320	880	171	190	170	220	440	320	470	330
Na	**[mg/L]**	350	480	310	440	920	640	1100	1700	356	316	270	460	410	430	630	790
Mg	**[mg/L]**	11	9.6	6.4	10	25	30	39	65	12	11	11	13	20	13	29	21
P	**[mg/L]**	67	91	78	150	200	160	320	750	66	77	98	130	290	190	300	200
S	**[mg/L]**	100	93	71	140	210	190	240	440	103	91	73	95	180	120	190	120
K	**[mg/L]**	660	720	190	440	320	370	340	760	667	828	860	1100	1300	540	490	200
Ca	**[mg/L]**	15	14	7.6	12	39	41	51	60	18	16	16	17	16	9.8	28	23
V	**[µg/L]**	0.2	0.2	0.2	0.2	0.3	0.2	0.3	0.7	0.2	0.3	0.1	0.2	0.2	0.1	0.2	0.2
Cr	**[µg/L]**	0.5	0.5	0.6	<0.5	1.0	<0.5	<0.5	0.7	0.6	0.8	<0.5	0.7	<0.5	<0.5	<0.5	<0.5
Mn	**[µg/L]**	0.2	0.3	0.1	0.2	0.5	0.3	0.4	0.7	0.2	0.1	<0.1	0.4	0.1	0.1	0.1	0.1
Fe	**[µg/L]**	6.5	8.2	4.9	5.6	73	4.7	19	91	7.4	7.8	1.3	7.1	3.1	5.8	2.4	3.0
Co	**[µg/L]**	<0.2	<0.2	<0.2	0.2	0.2	0.2	0.3	0.4	<0.2	<0.2	<0.2	<0.2	0.2	<0.2	0.2	<0.2
Ni	**[µg/L]**	<1	<1	<1	<1	<1	<1	1.9	3.2	1.3	<1	<1	<1	<1	<1	<1	<1
Cu	**[µg/L]**	4.1	4.4	4.2	3.5	3.6	3.5	5.2	8.0	3.2	4.4	3.9	3.7	5.7	3.1	2.7	2.1
Zn	**[µg/L]**	11	8.5	9.8	19	26	23	31	110	24	17	13	11	20	17	39	20
As	**[µg/L]**	2.9	2.3	2.3	3.3	2.9	3.4	3.8	5.8	420	390	130	170	210	110	130	90
Se	**[µg/L]**	6.5	7.4	6.4	9.5	11	8.5	12	23	9.4	7.2	8.6	8.7	10	8.9	11	8.2
Rb	**[mg/L]**	0.5	0.5	0.3	0.4	0.2	0.3	0.3	0.7	0.4	0.6	0.6	0.7	1.0	0.4	0.4	0.2
Sr	**[µg/L]**	36	33	19	32	81	93	120	190	48	44	42	43	48	35	86	68
Mo	**[µg/L]**	4.1	2.8	1.8	2.8	1.8	2.0	2.5	9.6	2.9	2.9	3.2	3.2	4.3	1.4	3.5	4.4
Cd	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Sb	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Te	**[µg/L]**	<0.2	0.2	<0.2	<0.2	0.2	<0.2	0.2	0.4	<0.2	<0.2	0.3	<0.2	0.2	0.2	<0.2	<0.2
Ba	**[µg/L]**	1.3	1.4	1.3	1.9	2.2	2.7	3.2	4.1	1.8	1.8	1.2	1.3	0.9	0.7	1.3	1.3
Hg	**[µg/L]**	0.8	0.8	0.7	1.0	0.5	0.3	0.6	1.6	0.6	1.0	0.4	0.3	0.3	0.4	0.4	0.4
Pb	**[µg/L]**	1.5	1.3	1.3	1.5	1.5	1.3	1.7	1.8	1.7	1.6	1.0	1.0	<1	<1	<1	<1
Bi	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
U	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 34 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 5/7 samples 32401 - 42302

Sample		32401	32402	32403	32404	32501	32502	32503	32504	32505	42202	42203	42204	42205	42206	42301	42302
USG		1.012	1.009	1.007	1.006	1.004	1.007	1.008	1.004	1.007	1.026	1.016	1.024	1.007	1.014	1.024	1.005
Li	**[µg/L]**	370	13	15	22	13	24	410	11	18	360	14	19	4.4	10	360	3.7
B	**[µg/L]**	1400	600	550	410	240	530	1000	230	400	2400	910	1400	230	500	2100	220
Na	**[mg/L]**	1900	590	560	800	460	750	970	440	1100	1600	1200	3400	1200	4700	2900	410
Mg	**[mg/L]**	55	24	40	24	15	25	39	12	23	160	130	130	31	69	150	31
P	**[mg/L]**	680	310	210	200	150	360	340	120	310	970	490	870	360	480	2300	170
S	**[mg/L]**	390	390	300	250	120	270	300	140	250	1100	660	1000	200	340	920	160
K	**[mg/L]**	1200	1800	460	270	130	460	370	790	1600	3600	1300	1300	230	560	900	210
Ca	**[mg/L]**	31	18	36	36	26	35	45	14	30	360	320	360	79	190	270	79
V	**[µg/L]**	0.8	0.4	0.3	0.2	0.3	0.2	0.4	0.2	0.5	1.2	0.6	1.0	0.4	1.0	0.9	0.2
Cr	**[µg/L]**	0.8	<0.5	<0.5	0.6	0.9	<0.5	<0.5	<0.5	0.8	0.6	<0.5	<0.5	<0.5	0.7	0.6	<0.5
Mn	**[µg/L]**	0.4	0.2	0.7	0.3	2.2	0.1	0.2	0.1	0.7	0.7	0.5	0.3	0.2	0.3	0.5	0.3
Fe	**[µg/L]**	110	11	23	7.8	39	4.5	110	2.2	12	95	13	14	5.7	9.5	100	9.2
Co	**[µg/L]**	0.3	0.3	<0.2	0.2	0.4	<0.2	0.2	<0.2	0.2	0.3	0.2	0.4	0.2	0.5	1.4	0.2
Ni	**[µg/L]**	2.7	2.1	1.3	1.5	32	<1	2.8	<1	8.2	4.2	3.0	4.1	1.6	2.7	6.0	1.7
Cu	**[µg/L]**	7.7	6.5	4.6	5.0	110	4.2	3.9	2.5	29	23	12	22	4.9	9.4	26	6.5
Zn	**[µg/L]**	800	78	53	61	160	42	58	20	69	563	286	260	90	79	830	160
As	**[µg/L]**	190	140	88	58	35	73	66	32	61	12	7.9	9.7	3.1	5.5	17	110
Se	**[µg/L]**	23	13	11	11	10	13	14	8.3	14	54	30	47	10	20	57	12
Rb	**[mg/L]**	1.1	1.4	0.4	0.2	0.1	0.4	0.3	0.5	1.1	2.6	0.9	1.3	0.2	0.4	1.0	0.2
Sr	**[µg/L]**	110	63	110	98	62	96	120	40	79	410	317	379	80	160	340	81
Mo	**[µg/L]**	25	17	12	21	11	21	19	7.4	12	53	52	94	17	36	150	17
Cd	**[µg/L]**	0.2	0.1	<0.1	0.1	0.7	<0.1	0.1	<0.1	0.3	0.2	0.2	0.1	0.1	0.1	0.4	0.1
Sb	**[µg/L]**	0.1	<0.1	0.1	<0.1	0.2	<0.1	0.1	<0.1	0.1	0.1	0.1	0.1	<0.1	<0.1	0.1	<0.1
Te	**[µg/L]**	0.3	<0.2	0.2	0.2	0.2	<0.2	<0.2	<0.2	0.2	0.4	0.2	0.3	0.3	0.5	0.4	<0.2
Ba	**[µg/L]**	1.9	0.9	2.0	1.4	2.7	1.4	1.1	0.7	1.8	4.8	18	5.9	2.0	3.7	5.3	1.7
Hg	**[µg/L]**	0.9	0.8	0.5	0.4	0.3	0.3	0.5	0.4	0.6	0.7	0.4	0.4	0.4	0.5	0.9	0.5
Pb	**[µg/L]**	<1	<1	<1	<1	10	<1	<1	<1	3.6	2.3	<1	1.3	<1	<1	1.5	<1
Bi	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	1.5	<0.1	<0.1	<0.1	0.3	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
U	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 35 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 6/7 samples 42303 - 52201

Sample		42303	42304	42305	42306	42307	42401	42402	42403	42404	42405	42406	42407	42408	42501	42502	52201
USG		1.007	1.017	1.013	1.019	1.018	1.025	1.022	1.005	1.010	1.020	1.006	1.006	1.012	1.021	1.023	1.020
Li	**[µg/L]**	5.1	10	9.8	20	16	370	16	3.9	9.1	16	3.5	3.1	6.5	410	15	380
B	**[µg/L]**	290	680	580	800	640	1500	830	180	440	760	170	250	600	2000	1400	1300
Na	**[mg/L]**	1400	2800	1200	1200	2900	1500	590	230	670	3200	850	1600	3400	3100	2000	3300
Mg	**[mg/L]**	42	62	46	52	96	110	83	22	55	61	18	17	34	75	110	67
P	**[mg/L]**	120	330	330	1200	740	2200	940	79	270	1200	160	75	360	1600	1100	1400
S	**[mg/L]**	190	490	450	660	710	1400	1000	170	420	800	160	150	360	770	940	1100
K	**[mg/L]**	840	3000	1500	2800	2000	960	3300	270	580	2300	230	360	1100	1400	3700	2500
Ca	**[mg/L]**	140	200	140	150	260	240	220	61	150	170	52	59	110	150	280	190
V	**[µg/L]**	0.6	1.3	0.6	0.7	0.9	0.7	0.6	0.1	0.3	0.8	0.4	0.3	0.8	1.0	1.0	1.4
Cr	**[µg/L]**	0.7	0.6	<0.5	0.9	0.8	1.0	0.8	<0.5	<0.5	1.0	0.7	<0.5	1.2	<0.5	0.5	1.4
Mn	**[µg/L]**	1.1	0.4	0.1	0.7	0.9	1.2	0.6	<0.1	0.3	0.3	0.6	0.4	1.8	0.3	0.3	2.2
Fe	**[µg/L]**	14	12	9.7	15	22	130	18	3.2	16	13	14	8.2	23	130	24	230
Co	**[µg/L]**	0.3	0.4	0.3	0.8	1.4	1.5	0.8	0.2	0.3	0.7	0.2	<0.2	0.5	0.7	0.6	0.2
Ni	**[µg/L]**	11	3.4	2.9	5.3	4.4	5.3	5.7	1.9	3.5	4.6	2.0	1.5	8.7	4.6	6.2	-
Cu	**[µg/L]**	34	16	14	27	17	26	21	5.0	10.0	17	4.4	5.0	24	18	20	15
Zn	**[µg/L]**	160	320	260	460	520	800	560	90	160	280	100	50	160	450	560	150
As	**[µg/L]**	1300	4100	2300	3200	1600	1700	1100	120	280	550	95	84	200	370	550	12
Se	**[µg/L]**	14	38	36	43	37	58	55	14	22	43	11	9.7	20	45	56	40
Rb	**[mg/L]**	0.5	1.9	1.3	2.4	1.5	1.1	2.7	0.2	0.6	2.0	0.2	0.2	0.6	1.3	3.0	2.2
Sr	**[µg/L]**	120	230	160	220	270	320	280	68	160	210	61	59	120	200	320	180
Mo	**[µg/L]**	16	40	34	45	43	59	48	9.7	26	51	8.8	6.6	14	35	40	100
Cd	**[µg/L]**	0.5	0.2	0.1	0.2	0.2	0.2	0.2	<0.1	0.1	0.1	<0.1	<0.1	0.3	0.2	0.2	0.4
Sb	**[µg/L]**	0.1	0.1	0.1	0.1	0.2	0.2	0.1	<0.1	<0.1	0.1	0.1	<0.1	0.1	0.1	0.1	0.2
Te	**[µg/L]**	<0.2	0.2	<0.2	0.4	0.6	0.3	0.2	<0.2	<0.2	0.3	<0.2	0.3	0.2	0.4	0.3	0.2
Ba	**[µg/L]**	3.2	3.8	3.0	7.1	4.7	4.7	4.2	1.1	2.3	3.0	2.2	1.0	4.0	3.6	4.6	4.3
Hg	**[µg/L]**	0.3	0.7	0.4	0.4	0.4	0.7	0.6	0.8	0.2	0.4	0.5	0.4	0.7	0.5	0.6	4.1
Pb	**[µg/L]**	4.5	1.2	<1	1.5	1.4	2.1	1.6	<1	1.1	1.2	<1	<1	3.3	<1	<1	1.4
Bi	**[µg/L]**	0.2	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.4	<0.1	<0.1	0.1
U	**[µg/L]**	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 36 USG and not normalized concentrations of 29 elements in human urine samples, measured with ICPMS, As values are not typical for urine because of the ingestion of 2 mg As, part 7/7 samples 52202 - 52502

Sample		52202	52203	52204	52205	52301	52302	52303	52304	52305	52401	52402	52403	52404	52405	52501	52502
USG		1.020	1.024	1.020	1.005	1.020	1.008	1.015	1.009	1.002	1.017	1.018	1.020	1.008	1.021	1.018	1.020
Li	**[µg/L]**	12	20	13	3.3	340	5.4	13	8.0	2.4	460	45	44	20	420	37	33
B	**[µg/L]**	480	840	790	160	1300	280	720	430	99	1900	910	1100	510	2100	1700	1500
Na	**[mg/L]**	4100	4600	2800	680	2200	790	1700	1800	250	2500	5500	4100	760	2500	2300	3200
Mg	**[mg/L]**	16	32	58	7.8	61	17	29	22	5.4	80	45	59	19	48	100	75
P	**[mg/L]**	1200	1500	1100	300	1700	220	660	500	110	1500	770	900	410	1700	1800	820
S	**[mg/L]**	880	990	690	170	890	260	560	340	79	750	690	620	280	1200	1000	730
K	**[mg/L]**	6700	6200	930	180	1100	590	2300	1100	100	1000	3100	3200	720	3900	1300	1600
Ca	**[mg/L]**	52	140	270	33	200	74	110	78	22	250	170	200	58	140	210	190
V	**[µg/L]**	1.9	1.7	0.8	0.3	0.8	0.4	0.7	0.6	0.1	0.9	1.6	1.4	0.3	0.9	0.7	0.9
Cr	**[µg/L]**	2.4	0.8	<0.5	0.5	0.5	1.9	<0.5	0.5	<0.5	0.6	2.1	1.4	<0.5	1.8	0.6	1.3
Mn	**[µg/L]**	4.4	1.1	1.0	0.5	0.9	2.2	1.1	1.3	1.0	2.2	3.8	2.9	2.1	2.5	2.4	2.6
Fe	**[µg/L]**	71	23	78	6.3	92	33	10	12	5.1	100	38	55	15	120	17	13
Co	**[µg/L]**	0.5	0.3	0.3	<0.2	0.4	0.2	0.3	0.2	<0.2	0.3	0.4	0.4	0.2	0.5	0.4	0.3
Cu	**[µg/L]**	210	17	10	3.3	14	8.3	14	5.5	2.3	13	12	13	6.7	22	12	12
Zn	**[µg/L]**	570	330	800	70	380	230	230	110	30	360	410	270	120	410	400	1200
As	**[µg/L]**	9.4	13	12	4.6	14	770	2000	420	63	500	310	290	89	240	220	210
Se	**[µg/L]**	37	39	32	9.3	36	14	29	15	7.9	34	38	43	19	39	39	39
Rb	**[mg/L]**	4.5	4.2	0.8	0.2	1.2	0.6	1.8	0.8	0.1	0.9	1.9	2.6	0.7	3.2	1.3	1.5
Sr	**[µg/L]**	68	170	240	35	200	80	140	87	25	240	170	200	89	200	270	230
Mo	**[µg/L]**	80	71	16	2.2	13	4.8	11	6.7	3.3	54	40	28	5.8	25	42	35
Cd	**[µg/L]**	0.6	0.3	0.1	<0.1	0.2	0.1	0.2	<0.1	<0.1	0.1	0.2	0.3	<0.1	0.3	0.3	0.2
Sb	**[µg/L]**	0.2	0.1	0.1	<0.1	0.1	0.2	<0.1	<0.1	<0.1	0.1	0.1	0.1	<0.1	0.1	0.1	0.1
Te	**[µg/L]**	0.3	0.5	0.2	<0.2	0.2	0.2	0.2	0.2	<0.2	0.3	0.3	0.2	<0.2	0.4	0.3	0.2
Ba	**[µg/L]**	3.4	4.1	4.4	1.2	4.1	3.8	3.3	1.7	0.8	4.6	4.6	4.6	2.5	4.6	5.0	4.2
Hg	**[µg/L]**	5.7	3.5	2.7	0.5	5.2	1.5	3.2	1.4	0.5	2.6	3.8	3.6	1.3	3.2	5.2	3.6
Pb	**[µg/L]**	15	1.1	<1	<1	<1	<1	<1	<1	<1	<1	<1	1.3	<1	1.7	<1	<1
Bi	**[µg/L]**	0.7	0.1	<0.1	<0.1	<0.1	0.1	<0.1	<0.1	<0.1	<0.1	<0.1	0.1	<0.1	0.1	0.1	0.1
U	**[µg/L]**	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1

Table 37 Results of the cationic arsenic speciation analysis, measured on a Zorbax 300-SXC column with a 20 mM ammonium formate buffer (pH 2.5) as mobile phase with HPLC-ICPMS

Sample	As(III), As(V), MA [µg/L]	DMA [µg/L]	AB [µg/L]	Sample	As(III), As(V), MA [µg/L]	DMA [µg/L]	AB [µg/L]
12201	3.1	3.5	3.2	32303	<1.0	<1.0	410
12202	1.9	4.3	2.8	32304	<1.0	<1.0	370
12203	<1.0	2.1	3.4	32305	<1.0	<1.0	220
12204	2.1	4.6	3.6	32306	<1.0	<1.0	170
12301	1.8	4.0	2.5	32307	<1.0	<1.0	210
12302	<1.0	<1.0	960	32308	<1.0	<1.0	110
12303	<1.0	<1.0	790	32309	<1.0	<1.0	130
12304	<1.0	1.0	120	32310	<1.0	<1.0	88
12305	<1.0	<1.0	64	32401	1.2	2.1	190
12306	<1.0	1.1	88	32402	<1.0	1.7	140
12401	<1.0	<1.0	330	32403	<1.0	<1.0	87
12402	<1.0	<1.0	270	32404	<1.0	<1.0	56
12403	<1.0	<1.0	200	32501	<1.0	<1.0	32
12404	<1.0	<1.0	280	32502	<1.0	<1.0	71
12405	<1.0	2.9	230	32503	<1.0	1.1	63
12501	<1.0	<1.0	270	32504	<1.0	<1.0	30
12502	<1.0	8.7	220	32505	<1.0	1.5	56
22201	3.7	5.0	2.2	42202	2.0	5.0	1.6
22202	1.5	5.8	3.4	42203	1.3	3.4	2.1
22203	1.3	4.9	3.9	42204	1.9	4.4	1.3
22204	<1.0	2.1	1.2	42205	<1.0	<1.0	<0.5
22205	1.1	2.7	2.5	42206	1.1	2.0	<0.5
22206	1.5	4.8	3.7	42301	3.7	8.4	1.2
22207	<1.0	1.1	0.9	42302	<1.0	1.2	110
22208	<1.0	1.5	2.4	42303	<1.0	<1.0	1300
22301	2.0	3.8	2.0	42304	<1.0	<1.0	4100
22302	<1.0	<1.0	26	42305	<1.0	<1.0	2700
22303	<1.0	<1.0	500	42306	<1.0	<1.0	2800
22304	<1.0	<1.0	1370	42307	4.2	5.4	1400
22305	<1.0	2.0	1200	42401	10	7.4	1500
22306	<1.0	9.1	680	42402	6.7	5.3	1100
22307	<1.0	<1.0	90	42403	<1.0	<1.0	110
22308	<1.0	<1.0	160	42404	3.4	2.1	270
22309	<1.0	<1.0	110	42405	2.7	4.6	520
22401	8.8	8.4	490	42406	<1.0	<1.0	86
22402	<1.0	<1.0	490	42407	<1.0	<1.0	83
22403	<1.0	5.5	270	42408	<1.0	2.1	190
22404	<1.0	1.1	91	42501	4.7	7.8	350
22405	<1.0	1.4	130	42502	5.7	6.9	500
22406	<1.0	1.3	110	52201	4.4	2.7	0.5

Sample	As(III), As(V), MA [µg/L]	DMA [µg/L]	AB [µg/L]	Sample	As(III), As(V), MA [µg/L]	DMA [µg/L]	AB [µg/L]
22407	1.1	2.3	200	52202	2.5	2.2	<0.5
22408	<1.0	2.5	180	52203	3.0	5.5	<0.5
22501	1.1	3.2	120	52204	2.6	5.6	<0.5
22502	<1.0	11	200	52205	<1.0	<1.0	<0.5
22503	<1.0	<1.0	270	52301	3.1	6.4	<0.5
32201	<1.0	1.9	1.9	52302	<1.0	<1.0	810
32202	<1.0	1.9	0.9	52303	1.0	2.6	1800
32203	<1.0	<1.0	<0.5	52304	<1.0	<1.0	410
32204	<1.0	<1.0	<0.5	52305	<1.0	<1.0	59
32205	<1.0	<1.0	<0.5	52401	3.4	4.6	490
32206	<1.0	<1.0	<0.5	52402	1.6	3.9	300
32207	<1.0	<1.0	<0.5	52403	3.5	4.2	280
32208	<1.0	<1.0	1.1	52404	1.9	3.2	200
32209	<1.0	<1.0	1.2	52405	4.5	4.8	210
32210	<1.0	<1.0	1.4	52501	2.1	2.9	210
32301	<1.0	<1.0	1.4	52502	<1.0	<1.0	98
32302	1.2	<1.0	1.4				